The Influence of Maternal Care on Stress-related Responses in Puppies, *Canis lupus familiaris*

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Abstract

Maternal care alters neurology and behaviour in mammals. However, little is known about the impact of maternal care in domestic dogs. Rapid neurological development occurs in dogs between postnatal days 3 and 16, yet maternal care within this period has not been thoroughly investigated. Knowledge of neurological development and maternal care is mainly derived from studies of white laboratory rats and, while numerous differences exist between rats and dogs, the physiological mechanisms underpinning the programming of stress-related behaviour are likely to be similar. This thesis sought to investigate maternal care in dogs and the impact it may have on stress-related behaviour in later life.

The methodology used to study maternal care in dogs is differing and therefore an aim of this thesis was to provide an overview of differences between litters while comparing sampling methodologies. Six litters of dogs were observed on postnatal days 3, 6, 9 and 12. Maternal care behaviours included dam presence, nursing, contact (frequent behaviours) and anogenital licking (infrequent behaviour). A 24-hour period was compared to five time sampling periods: 12-hour night (1800-0600 hours), 12-hour day (0600-1800 hours), and two sets of four fifteen-minute periods during: night (1800-0600 hours), day (0600-1800 hours) and anytime. A shorter sampling period (four fifteen-minute periods) was representative of the 24-hour period for frequent behaviours.

Maternal care behaviour impacts offspring response to stress later in life. An observational study was employed to determine whether maternal care behaviours differ between and within dog litters. Ten litters, including six of the litters above, were observed over postnatal days 3, 6, 9 and 12. Differences between dams were evident for all behaviours ($p<0.001$), while no differences within a litter were observed for any behaviour.

Isolation initiates fear in domestic animals, thus an isolation box can be used to determine stress-related behaviour. Forty-seven puppies (previously assessed for litter behaviour) were observed for activity and vocalisations, and had their heart rate recorded. Breed type and size, parity, and anogenital licking on day 6 were associated with stress-related behaviours. In particular, puppies that were licked more had a shorter latency to vocalise and a higher pre-test heart rate, although this was not expected. Differences in the stress axis responsible for stress-related behaviour and physiology, or the onset of fear-related behaviour, may be reason for these results.

Given that maternal care behaviour differed between dams, breeders were asked (using an online survey) whether they consider maternal care of bitches when selecting breed stock. There were 274 respondents representing all breed groups of the Australian National Kennel Club. Four components were found using Principal Components Analysis: Maternal Care, Offspring Potential, Dam Temperament, and Dam Genetics and Health. Maternal care was scored as more important by toy and hound groups, while breed priority was altered by the number of breeds kept and brachycephalic breeds. Maternal care should be considered by breeders when choosing stock to rebreed from, due to its potential impact on stress in puppies.

The findings of this thesis allow behavioural studies to employ a shorter sampling period, highlights maternal care differences in dogs, and allows a simple test for breeders to use to assess puppy behaviour.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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30.08.2016

Author contributions

This thesis contains three peer-reviewed articles (Appendix 2, 3 and 4). The articles are multi-authored but I am the lead author on each. I was responsible for writing the manuscripts and data analysis. Descriptions of the involvement of each author and their agreement to the inclusion of the article and manuscripts in this thesis are provided below.

Dr Susan Hazel, Professor Philip Hynd and Dr Bradley Smith provided advice and editorial assistance for all manuscripts. Dr Michelle McArthur provided advice, editorial assistance and statistical advice for the breeder survey. All authors supervised the development of the work presented for publication and assisted in editing the manuscript, in their respective article or manuscript. Dr Susan Hazel, Professor Philip Hynd, Dr Bradley Smith and Dr Michelle McArthur have given consent to present this article for examination towards the Doctor of Philosophy.

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Chapter 1. Introduction

1.1 Context

The domestic dog, *Canis lupus familiaris*, evolved and developed to live among humans. Currently, approximately 39% of Australian households own a dog (Animal Health Alliance, 2013). Human benefits of owning a dog include lowering depression levels (Brickel, 1984; Zasloff and Kidd, 1994), lower stress levels (Kidd and Kidd, 1999) and reducing the risk of coronary heart disease (Parslow and Jorm, 1992). Conversely, some studies have found no association between human health and companion animals (e.g. Wells and Rodi, 2000), while there is also evidence that dog companionship increases the level of depression in humans (Fritz et al., 1996; Antonacopoulos and Pychyl, 2010). Therefore, while the human-dog bond may be positive and dogs are an important part of the lives of humans, at times, the bond may become detrimental or negative leading to the dog being relinquished.

Between the 1st of July, 2014 and the 30th of June, 2015, almost 47,000 dogs were relinquished to RSPCA shelters within Australia. Of those relinquished, almost 15% (6,765) of dogs were euthanised, of these 4,700 dogs were euthanised due to behavioural problems (RSPCA, 2015). Anxiety is a common undesirable behaviour trait leading to dog relinquishment (Miller et al., 1996; Patronek et al., 1996; Salman et al., 2000; Segurson et al., 2005), but the number of dogs affected by anxiety is unknown. Anxiety results when an individual processes information and perceives it as a threat or danger to their safety or security (Beck et al., 1985). Indeed, anxiety is an essential emotion, but can become detrimental when the anxiety-response is inappropriate for a situation, reducing the individual's ability to adapt to the environment (Ohl et al., 2008). Anxiety is a secondary response to stress, i.e. a physical, psychological or environmental stimulus threatening the individual and in turn altering homeostasis (Moberg and Mench, 2000), as are separation anxiety and aggression (Rapee et al., 1996; Monnelly et al., 2003;

Gillespie et al., 2009). This thesis does not fully delve into the mechanisms of stress but a comprehensive review of the neuroendocrine mechanisms in response to stress has been documented (Tilbrook and Clarke, 2006).

There are two main responses to anxiety: physiological and behavioural. Physiological signs in the dog include increased salivation, respiration rate and heart rate, along with paralysis, vasomotor changes and gastrointestinal disturbances (Sherman and Mills, 2008). Behavioural signs in the dog include circling, pacing, panting, restlessness, immobility, changes in appetite and trembling (Sherman and Mills, 2008). The posture of the dog is also a potential indicator of stress; fear-related body posture includes ears drawn back, gaze aversion, tail tucking and, if aggression develops, the dog lowering its body stance (Bain, 2009). Short-term symptoms of anxiety include intermittent vomiting, diarrhoea, decreased appetite (Sherman and Mills, 2008), coprophagia (eating own faeces) (Hart et al., 2012), signs of inflammatory diseases (Sternberg et al., 1992), dermatoses (Dreschel, 2010; Landsberg et al., 2013), and/or pyoderma (Nagata et al., 2002). Short-term stressors, resulting in increased corticosteroid level, can lead to chronic frustration (Mills et al., 2013), nervousness, restlessness, increased barking, avoidance response, food guarding and startle response (Notari and Mills, 2011). Constant stressors can lead to detrimental long-term effects, including reduced reproductive success, decreased sperm quality in male dogs (Memon, 2007) and reduced breeding ability in bitches (Grundy et al., 2002). Chronic stressors alter the body's response to invading pathogens, affecting the immune system (Radek, 2010). Dogs in shelters experiencing acute or chronic stress have reduced system function (measured by cortisol and heart rate) (Beerda et al., 1998; Hennessy et al., 1998). There is some indication that anxiety may reduce the life span of a dog. A survey regarding deceased pets completed by 721 respondents found dogs with extreme fear of strangers had a six-month reduction in lifespan (Dreschel, 2010).

Undesirable behaviours in dogs can result from many factors, including genetics (Goddard and Beilharz, 1983; Saetre et al 2006; Morrow et al., 2015), environment (e.g. parental behaviour, husbandry methods, interaction with conspecifics, humans and new experiences) (Hetts et al., 1992; Jagoe and Serpell, 1996; Kobelt et al., 2007), and exposure to training and training methods (Jagoe and Serpell, 1996; Hiby et al., 2004; Rooney and Cowan, 2011). Many of these factors have been explored to better predict future desired traits for individuals kept for breeding, exhibition, work, recreation animals and household pets (Slabbert and Odendaal, 1999; Hennessy et al., 2001; Svartberg, 2002; Foyer et al., 2013). However, the success of accurately predicting a dog's response to stress remains low (Beaudet et al., 1994; Wilsson and Sundgren, 1998b; Seksel et al., 1999; Slabbert and Odendaal, 1999; Asher et al., 2013; Kutsumi et al, 2013; Riemer et al., 2014; Rocznik et al., 2015; Robinson et al., 2016). Studies determining future stress response have mainly focussed on juveniles, but few address the impact of experiences during early development, particularly interactions between dam and puppy.

The dog is an altricial species, where young are born deaf and blind, have limited movement and require the mother for survival (Kendrick et al., 1997). In altricial species the impact of maternal care and early postnatal environment may have marked effects on subsequent stress-related behaviours. A relationship between maternal care in puppyhood and anxiety development later in life was identified by Tiira and Lohi (2015), with dogs receiving less maternal care more likely to display later anxiety later in life. However, Tiira and Lohi (2015) surveyed owners and it is unlikely that owners can state with certainty the amount of maternal care the puppy received. A more accurate determination of mothering ability could be gained from surveys targeting breeders.

Direct evidence of a link between maternal behaviour and later puppy anxiety has only recently been documented (Foyer et al., 2016; Guardini et al., 2016). Foyer et al. (2016)

observed maternal behaviour of 22 German Shepherd litters during the first three postnatal weeks. The authors found the level of dam maternal care differed, as measured by dam duration in the box, lying in contact, nursing, licking and sniff/poke, and dams grouped according to whether they gave a low or high level of care. At 18 months of age puppies in the lower maternal care group displayed less social engagement (willingness to interact with humans), physical engagement (competiveness, hunting drive, prey drive and liveliness) and displayed aggression (sharpness and defence drive). However, in that study a total of 12-hours (for one hour every second hour) of maternal behaviour was observed on one day a week (see Chapter 3), while changes occurring across the week were not observed suggesting that maternal care behaviour may have not been effectively observed. Guardini et al. (2016) also documented the impact of maternal care on later puppy behaviour. Each puppy-dam dyad was recorded for 15 minutes in the morning for the first three postnatal weeks to determine duration of maternal care (contact, licking, anogenital licking and nursing) received. At approximately eight weeks old, the puppies undertook an isolation test where they remained alone for three minutes. Behaviours observed in isolation included stress behaviours, vocalisations, non-social behaviour and other behaviours. The researchers found that increased maternal care resulted in an increase in exploration, longer latency to first yelp and a decrease in interaction with the enclosure, movement not considered explorative and whining/yelping. This suggests that puppies receiving more maternal care are less stressed during isolation. However, the amount of maternal care given to the puppy was not documented in that study, and each of the maternal care behaviours were aggregated using principal components analysis, rather than documenting the effect of each maternal behaviour on puppy response later in life. To thoroughly understand maternal care behaviour, dam behaviour may need to be observed more frequently during the first two week postnatal period, as this is the critical period for hypothalamic-pituitary-adrenal axis development in rats (Levine and Lewis, 1959; Meaney and Aitken, 1985; Myers et al., 1989). It would also be useful to predict an animal's future anxiety behaviour when it is younger (i.e. less than one year of age) to allow invention to occur earlier. Although Tiira and Lohi (2015), Foyer et al. (2016) and Guardini et al. (2016) highlight a link between maternal care and later anxiety in dogs, our understanding of maternal care in domestic dogs, in particular in the critical first two week postnatal period, remains limited. Maternal care as a predisposing factor has been described in altricial species, but is a relatively new concept in relation to dogs and anxiety later in life. The influence of behaviour in puppies is complex with many areas affecting behaviour, and thus this thesis delves into a small area of the issue.

1.2 Aims and objectives

The first aim of this thesis was to gain a thorough understanding of distribution and frequency of maternal behaviours. This was conducted by observing between- and within-litter differences of domestic dogs, with a particular focus on duration of dam presence, nursing, contact and anogenital licking.

The second aim was to determine whether maternal behaviour influenced the puppy's stress-related behaviour later in life. To determine stress-related behaviours, eight-week old puppies were placed in an isolation box to impose a stressful event.

The third aim was to understand dam selection, in particular whether maternal care was considered a factor by Australian purebred dog breeders. This was achieved by an Australia wide survey of dog breeders. Research in this area is limited and therefore it was not known whether maternal care is taken into consideration when selecting dams for breeding.

This thesis specifically examines maternal care behaviours and the impact they may have on puppies, in particular stress-related behaviour. However, this thesis does not 1) determine what constitutes a good mother, 2) determine dam variables associated with selecting a good mother, or 3) the impact of mothering.

1.3 Research approach

This thesis is comprised of four research studies, namely a methodological study, two experimental studies, and a survey of Australian purebred dog breeders.

To determine the methodology used to describe maternal care (in Chapter 4), several behavioural coding strategies were employed and compared. Six litters of domestic dogs were recorded, observed and coded for a 24-hour period across postnatal days 3, 6, 9 and 12. This period was then used for 1) a 12-hour night, 2) a 12-hour day, and 3) three one hour periods (one during the day, one during the night time and one throughout the 24-hour period) comprised of four 15-minute periods. Duration of maternal behaviour was compared between methods.

In Chapter 4, 10 litters of domestic dogs were studied. The duration of dam presence in the whelping box, nursing and contact (puppy lying in contact with another puppy or dam) was documented. Duration, as well as frequency, was explored for anogenital licking. The interactions were observed across four days within the first two week postnatal period.

The second experimental trial is documented in Chapter 5, using the same litters presented in Chapter 4. This trial was designed to determine whether there is a link between maternal care and future puppy behaviour. The trial utilised 47 puppies aged 7-8 weeks and assessed behaviour and physiology in response to an isolation test.

In Chapter 6, a survey was used to explore attitudes of Australian purebred dog breeders. An online cross-sectional survey was designed to understand selection of dam and sire for breeding stock. Importantly, the survey indicated the importance placed on maternal care by pedigree dog breeders.

1.4 Relevance of the thesis

This thesis provides a significant addition to the field of maternal behaviour in canines. First, it provides a comprehensive insight into maternal care behaviour in domestic dogs. Second, relationships between maternal care in the first two postnatal weeks and puppy stress behaviour at seven weeks of age were explored. Finally, the use of a breeder survey provides insight into the selection practices of Australian purebred dog breeders, in particular priorities influencing dam selection.

The purpose of this thesis was to determine an appropriate time sampling method that represented maternal care behaviour accurately. Despite knowledge gained from studies surrounding frequent/infrequent and the appropriateness, this advice has been largely ignored in studies of maternal care in dogs. Ultimately, the objective of this thesis was to use previously reported methods (frequent/infrequent time sampling) to compare maternal care behaviour in domestic dogs and the impact it may have on stress-related behaviour in puppies. The knowledge gained in this research builds upon the small body of work which already exists. Furthermore, this thesis provides knowledge to breeders about the importance of maternal care and dam behaviour. This information may be useful in helping breeders choose more attentive dams, which may lead to a reduced number of puppies predisposed to anxiety.

Chapter 2. The impact of maternal care in dogs on stress-related behaviour later in life

The previous Chapter outlined the need for a deeper understanding of maternal care behaviour in domestic dogs due to its link with stress and anxiety later in life. It also highlighted the importance of understanding the link between early experiences and later behaviour. This current Chapter contains a review of the literature relating to maternal care and its implications for future offspring behaviour, in particular the physiological and behavioural repercussions of differential maternal care behaviour.

2.1 Introduction

Parents not only provide the genetic material for their offspring, but are a fundamental part of the offspring's environment. Thus, parents may be a major contributor to the development of their young. One of the earliest pioneers in maternal care research was psychologist Harry Harlow. He was instrumental in developing an understanding about the needs of infants, using rhesus monkeys as his experimental model. In Harlow's experimental trials, infant rhesus monkeys were removed from their mothers at a very young age, and isolated in cages where they were observed clinging to cloth at the bottom of their cage (Harlow, 1958). Infants were then presented with two types of surrogate mothers: a cloth covered mother and a mother covered in wire-mesh with a milk bottle attached to feed the infant (Harlow, 1958). The infants would feed from the mother with the milk but once they finished eating, the infants would move to the cloth-covered mother and, in total, the infants spent more time on the cloth-covered surrogate mother. The influence of the mother on infant behaviour was profound. Infants reared by mothers or surrogate mothers were observed in play behaviour more often than infants reared in a group or pair-reared (Chamove et al., 1973). As adults, the offspring reared in a group or pair-reared were more hostile and withdrew from others more often (Chamove et al., 1973). Social development is also impaired when infants are not raised by their mothers. Immediate and prolonged negative behaviours, such as agitated locomotive behaviour and a decrease in play and activity, are observed when mother and infant separate (Seay et al., 1962; Seay and Harlow, 1965; Hinde and Spencer-Booth, 1971). For the young of altricial species, the early social and physical environment is determined largely by interactions with the mother (Francis and Meaney, 1999). Even subtle variations in maternal care can impact the development of offspring, as will be discussed within this Chapter (Liu et al., 1997; Caldji et al., 1998; Caldji et al., 2000). The impact of early experiences and the environment on future behaviour may also affect dogs.

2.2 Maternal care in dogs

In a natural birth, the dam licks the placenta off the newly-born puppies, eating the placental remains, and biting the puppy's umbilical cord (Jack and Watson, 2008). Puppies are likely to nurse while the dam is still whelping other puppies in the litter. From birth until the puppies are approximately four weeks old, the dam is needed to stimulate urination and defecation (through anogenital licking), feed the puppies, and provide a heat source to allow the puppies to maintain a stable body temperature (Walker, 2010). The neonatal period, also known as the primary period, begins at birth until postnatal day 12 (Scott and Fuller, 1965), or between postnatal days 3-16 (Battaglia, 2009). This period is important as puppies are sensitive to thermal and tactile stimulation, motion and locomotion (Fox, 1972; Dunbar, 1979; Hoffman et al., 2004) and therefore a detrimental stimulus to the puppy within this period may alter the animal's behaviour later in life. Between postnatal days 3-16 there is rapid neurologic growth and development in puppies (Battaglia, 2009). While the puppies are relatively young and immature, the dam will protect them from other animals and unknown humans posing a threat.

By the end of the fourth postnatal week, puppies have coordinated walking, autonomic thermoregulation, visual depth perception and begin voluntary consumption of moistened solid food (Lawler, 2008). The dam may regurgitate food when the puppies are begging, although it is not common (Lord et al., 2013). Between this period and five weeks postnatally, the dam starts to frequently walk away from the whelping area, signalling weaning has commenced (Wilsson, 1984). The socialisation period begins pre-weaning when the puppies are three weeks of age and continues until approximately 12-14 weeks postnatal (Battaglia, 2009). In the socialisation period, the interaction of the puppy with novel objects, animals and individuals allows familiarity with those components later in the puppy's life (Scott et al., 1974). There is conflicting evidence about the specific time at which socialisation ends as some breeds have delayed onset of particular behaviour. For example, there are differences in the first appearance of fear-related avoidance behaviour between German Shepherds (39.4 ± 6.5 days), Cavalier King Charles Spaniels (55.1 ± 3.1 days) and Yorkshire Terriers (42.2 ± 2.5 days) (Morrow et al., 2015). Further work is needed to better define the socialisation period of different breeds.

Compared to wild canine species, domestic dogs are considered to have a reduced inclination for maternal behaviour (Lord et al., 2013) and therefore human intervention may be required for puppy survival. Although dam-puppy interactions have been documented (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Wilsson, 1984; Grant, 1987; Malm and Jensen, 1997; Nagasawa et al., 2014; Guardini et al., 2015; Foyer et al., 2016) there is yet to be a comprehensive description and documentation of these behaviours within the neonatal period. Further discussion of these behaviours are described below.

2.2.1 *Methodology used to study maternal behaviour in dogs*

Of the reports documenting maternal care in domestic dogs, differing and potentially-limited methodologies for assessing maternal behaviours have been used, leaving many questions unanswered. Altmann (1974) recognized the need to be rigorous in applying appropriate methodologies to study behaviour. For example, distinguishing between an 'event' (an instantaneous behaviour such as an animal lies down) and a 'state' (a behaviour with an appreciable duration such as an animal is lying) can impact the frequency of the behaviour observed (Altmann, 1974). The onset and termination of the session is also important; the length of the session affects the behaviours observed and observer fatigue can influence results. Comments made by Altmann (1974) in regard to observer fatigue relate to direct observation as opposed to video recordings which are now widely employed in observational studies. Video footage may enhance the duration and accuracy of observations, lessens observer fatigue, and allows the observer to accurately describe behaviours occurring simultaneously.

Methods for observational sampling have been described (Altmann, 1974) and sampling methods relevant to maternal behaviour are outlined below. Different sampling methods have been used in previously published studies of maternal behaviour in the dog within the first two postnatal weeks, as shown in Table 1 (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016). *Ad-libitum sampling* involves the researcher recording the animal's behaviour at non-elected sampling periods (Altmann, 1974). This method allows only the behaviour of individuals in view to be recorded, while behaviours portrayed by others or at another time would not be. This technique is commonly used during the first stages of behaviour quantification. Behaviours recorded using this method are not standardised for length or individuals and therefore it becomes difficult to compare behaviour between individuals, groups, and studies (Altmann, 1974). Rheingold (1963) and Scott and Fuller (1965) used *ad-libitum* sampling to determine nursing. Although there were similarities in the total duration of nursing observed, the minimum amount of nursing observed differed. Differences may be due to mothering ability, breed differences or methodology.

The *focal-animal* method of sampling is used to record all occurrences of a specific action of one individual or group of individuals. The length of each observation period and the time the individual/group is within view is recorded (Altmann, 1974). This method is recommended to determine the percent of time, rates and durations, chronological constraints (where time may alter the behaviour observed between individuals) and neighbour relationships for a particular behaviour (Altmann, 1974). It is not intended to determine non-frequent behaviours, unless there is a sufficiently long enough observation period to provide accurate results. Results presented by Rheingold (1963) for anogenital licking may be less precise as the time for observation (15 minutes, four times a day) may not have been long enough to determine infrequently occurring behaviours (Powell et al., 1977; Saudargas and Zanolli, 1990). Foyer et al. (2016) also used the focal-animal method. Their results are more likely to reflect the true maternal care behaviour of both frequent and infrequent behaviours as the observation period was longer (every second hour for an hour, once a week). However, the behaviours were totalled and then divided by the litter size, which may not reflect the true interaction each puppy had with the dam.

Instantaneous and scan sampling is recommended to determine the percent of time a particular behaviour is occurring (Altmann, 1974). This method requires an observer to record the behaviour of an individual during preselected sessions, and is useful in calculating the percent of time the behaviour is occurring within the sample period. Differences in nursing, contact and anogenital licking between studies using instantaneous and scan sampling method (Table 1) may explain variations in results, as both the length and the times chosen for observation differ between studies.

Studies observing maternal care behaviour within the first two postnatal weeks (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016) are listed in Table 1. There are a number of different sampling methods used, as well as sampling periods and sampling durations, which may explain differences in results. A detailed comparison between previous studies observing maternal care in dogs is reported in Chapter 3 of this thesis.

Table 1: Maternal care methodology in domestic dogs comparing sampling method, sampling duration and behaviours observed

Paper	**Sampling method**	**Sampling period (postnatal days)**	**Sampling duration**	**Behaviours observed**
Rheingold, 1963	Ad libitum Focal-animal sampling	1-68	15 minutes periods x 4 times a day between 0800 and 1800 hours	1. Contact between dam and litter 2. Number of pups in contact with the dam 3. Nursing duration 4. Number of pups suckling 5. Licking of the pups by the dam 6. Number of times the dam went to the pups 7. Number of times the dam went away from pups 8. Number of times pups went to dam 9. Number of times the dam punished the pups 10. Time the dam spent in the whelping box
Scott and Fuller, 1965	Ad lib	1-49	10 minutes observations, weekly	1. Nursing behaviour 2. Retrieving test 3. Weaning test
Korda and Brewinska, 1977a, 1977b	Instantaneous and scan sampling	2-4 and 13-16	14 hour period with constant sampling throughout, on postnatal day 1-4 and 13-16	1. The amount of time spent by the bitch in direct tactile contact with the puppies 2. Number of acts of licking the puppies by the bitch 3. Number of feeding acts 4. Total time of feeding 5. Puppy feeding posture

Grant, 1987	Instantaneous and Scan sampling	1-21	One hour per day spread across a 24-hour period on days 1-4, 7-10 and 12-21	1. Total time feeding 2. Feeding frequency 3. Average length of feeds 4. Time bitch spent away from pups 5. Puppy cleaning frequency 6. General observations (bitch position while feeding, puppy action while feeding, cleaning)
Guardini et al., 2015	Instantaneous and Scan sampling	1-21	15-minute video recording	1. Contact mother to pup 2. Nursing 3. Licking 4. Licking anogenital area
Foyer et al., 2016	Focal-animal	1-21	Every second hour, for an hour, once a week	1. Mother in Box 2. Lying in contact 3.Nursing 4. Licking 5. Sniff/poke

2.2.2 Management practices and puppy development

Management of breeding practices may alter the dam's behaviour towards her litter. A common practice for dog breeders is to remove a puppy from their littermates and the dam, and hold the puppy for a short period of time (Battaglia, 2009). In a study to assess the impact of handling, 43 dogs were separated into four groups: non-handled puppies raised in family, handled puppies raised in family, non-handled puppies raised in a professional breeding kennel, and handled puppies raised in a professional breeding kennel. Handled puppies were removed from the litter between postnatal days 3 and 21, massaged and turned on their back where an abdominal massage was undertaken before the puppy was placed back into the litter (Gazzano et al., 2008). At eight weeks of age, the puppies were placed into a new environment, isolated from their mother, littermates, and the breeder. The outcome of the test indicated that handled puppies spent significantly more time exploring the environment compared to non-handled puppies, while puppies raised in a breeding kennel had a longer latency to yelp and spent less time in vocalisation (Gazzano et al., 2008). A similar principle was used by the US military in developing the Bio Sensor stimulation exercises. The following exercises were undertaken on puppies: tactile stimulation, head held erect, head pointed down, supine position and thermal stimulation. Puppies handled and stimulated once a day were more active and exploratory than non-stimulated littermates during competitive situations (Fox, 1972).

In the previous paragraph, the importance of human interaction with puppies was explored. The importance of maternal care behaviour however, is limited. Literature from rats suggests that the type of maternal care given to the offspring can alter the behaviour of offspring later in life (e.g. a shorter swim path to a target (Liu et al., 2000)). To be able to draw parallels between rats and dogs, first a comparison of maternal care between the two species must be made.

2.3 Interspecific differences between the dog and the rat

Dogs and rats are altricial species and may be comparable in relation to maternal care and offspring development. The gestation of the rat is approximately 21 days (Eleftheriades et al., 2014) and frequently the mother is both lactating and pregnant at the same time (Prager et al., 2010). The domestic dog has a normal gestational period of 64-66 days (Wells and Hepper, 2006) and the bitch comes into oestrous approximately every seven months (Macdonald and Carr, 1995). However, the age the bitch first comes into oestrous differs with breed size; small females (8-15kg) come into season at seven months of age while larger females (30-40kg) can come into season as late as eighteen months (Lord et al., 2013).

Dogs and rats are comparable in that they both, usually, have more than one offspring in their litter. Litters of rats range from 6-12 pups (Viana et al., 2013) while in seven breeds of dog with over 100,000 litters recorded, average litter size ranged from 3.5 ± 1.7 pups in West Highland White Terriers to 6.3 ± 3.1 pups in Leonbergers (mean ± standard deviation) (Leroy et al., 2015). With a smaller number of litters (10,810) but a larger variety of breeds, an average overall litter size at birth was calculated to be 5.4 ±0.025 pups, with a range of 1-18 puppies (litter size included puppies born both alive and dead) (Borge et al., 2011). Similarities between litter sizes have been reported, as have physiological differences. The myelin sheath around axons of neurons is important in promoting neural conduction in the nervous system (Simons and Lyons, 2013). The rate of development of the myelin sheath differs between mammals. In dogs, the brain shows signs of myelin fibres in the posterior fossa, near the brainstem and cerebellum, at two weeks of age while secondary myelin branching is apparent after six weeks of age (Moit-Noirault et al., 1997). The myelin sheath is present within three days of birth in the rat, develops rapidly and is mature when the pup is two weeks old (Webster, 1971). Despite this difference in myelination, critical developmental events in rats and dogs occur at similar times, with the exception of auditory perception which is delayed in dogs (Table 2). Developmentally there is overlap between young of rats and dogs, thus factors influencing development in the rat, such as maternal care, are also likely to be seen in the dog.

Table 2: Critical events in the first two weeks of life of dogs and rats. Values are days postnatal.

Developmental event	Rat (postnatal day)	Dog (postnatal day)
Eyes open	14-15[a]	13[e]
Hearing	9[b]	19[e]
Smelling	By day 1[c]	Respond to chemosensory cues from birth[fg] Fully functional between 8-13[h]
Independently urinating and defecating	Mothers lick at high rates until 21 days, suggesting this is when they begin to undertake the process independently[d]	14-21[i]

a=Weisse, 1992, b=Feldman, 1992, c=Mendez-Gallardo and Robinson, 2011, d=Moore and Chadwick-Dias, 1986, e=Battaglia, 2009, f=Fox, 1971, g=Wells and Hepper, 2006, h=Scott et al., 1974 and i=Lawler and Chandler, 1992.

2.4 Maternal care in rodents

Rats are a particularly good model to determine possible links between maternal care and stress responses. The short development and lifespan (Holliday, 1989) allows researchers to follow rodents for a shorter period of time to determine behavioural differences across the animal's lifespan. Maternal behaviours include licking and grooming, nursing postures, retrieval of pups as well as the retrieval latency (how long the mother will take to retrieve the puppies which have moved away from the litter), nest building (Fleming and Rosenblatt, 1974), carrying the pups and blanket nursing, where the mother lies over the offspring with a low dorsal arch posture (Pryce et al., 2001). Retrieval of pups that have moved away from the mother and the lactating position are important maternal behaviours to allow a more successful attachment of the puppies to the nipples, and therefore ingestion of milk. The ideal dam posture is arched-back nursing, where the mother's hind feet are spread and the back is raised in an arch (Fleming and Rosenblatt, 1974). Variation in maternal care behaviours, such as licking, grooming and nursing position, have profound effects on the offspring's behavioural and neural phenotypes (Pan et al., 2014). Low licking/grooming and arched-back nursing (LG-ABN) mothers are those which are at least one standard deviation below the mean value ascribed to this behaviour while high LG-ABN mothers are classified as those mothers at least one standard deviation above the mean (Liu et al., 2000). Significant differences in maternal behaviours, in particular licking and grooming, were found during the first week postpartum with peak occurrences of licking and grooming observed within the first few postnatal days (Champagne et al., 2003). Factors such as litter size and sex ratio of the litter were not relevant in determining the mother's maternal behaviour (Champagne et al., 2003). In particular licking/grooming was not significantly affected by the size of the litter. However, observations were not coded for within a litter, and differences may be seen if within litter observations were recorded. Physiological changes in the brain, associated with altered maternal care, are well-described in the rat (Liu et al., 1997; Caldji et al., 1998; Caldji et al., 2000). At approximately postnatal day 21 weaning occurs in a laboratory setting (Inagaki et al., 2013; Mucellini et al., 2014; Williams et al., 2014).

2.4.1 Effect of maternal care in rodents

As both the rat and dog are multiparous, altricial species and their developmental stages are similar, it is likely that relationship between stress and maternal behaviour in the rat and dog are comparable. The physiological and behavioural responses in rats that occur during a novel or stressful situation in relation to the amount of maternal care received are described. The intention of this section is to provide an overview of the research. Further information can be sought in a recent comprehensive review on stress responses in rodents following high and low levels of maternal care (Anacker et al., 2014).

2.4.1.1 The hypothalamic pituitary adrenal (HPA) axis

A difference in the amount of maternal licking and grooming alters the offspring's response to novelty. Offspring with high LG-ABN mothers had a significantly reduced fear response to novelty compared to offspring of low LG-ABN mothers (Caldji et al., 1998). Pups licked more during the first 10 postnatal days showed a number of physiological differences including: reduced plasma adrenocorticotropic hormone, reduced corticosterone responses to acute stress, increased hippocampal glucocorticoid receptor (GR) messenger RNA expression, enhanced glucocorticoid feedback sensitivity and a decreased level of hypothalamic corticotropin-releasing hormone messenger RNA (Liu et al., 1997; Caldji et al., 1998). Some of these pathways are depicted in Figure 1. Physiological responses were significantly associated with maternal licking ($r>-0.6$) suggesting that maternal behaviour alters the hypothalamic-pituitary-adrenal response to stress in the offspring (Liu et al., 1997).

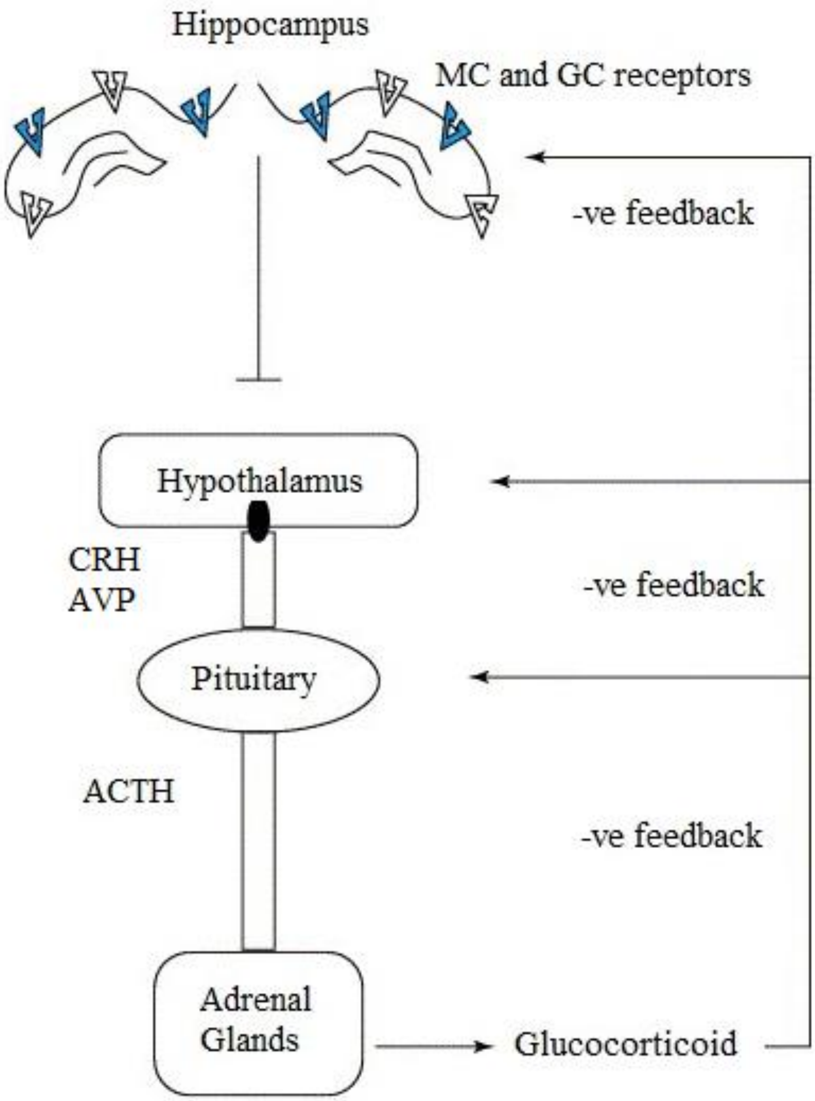


Figure 1: The hypothalamic-pituitary-adrenal (HPA) axis. Within the paraventricular nucleus of the hypothalamus, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are expressed and released in blood vessels. These hormones stimulate Adrenocorticotropic hormone (ACTH) resulting in glucocorticoid (corticosterone or cortisol, dependant on species) release from the adrenal glands. The glucocorticoid then acts via negative feedback on multiple pathways of the axis, including the mineralocorticoid (MC) and glucocorticoid (GC) receptors. Multiple steps within this pathway of pups can be altered by the frequency and duration of licking/grooming and nursing posture of the mother. Figure modified from Meaney and Szyf (2005).

2.4.1.2 Receptors and receptor binding in the Central and Peripheral Nervous System

Behaviours of the mother, in particular licking and grooming, alter a major neurotransmitter in the central nervous system, the $GABA_A$ receptor complex (Caldji et al., 2000). The complex is involved with binding molecules, such as benzodiazepines, which have antianxiety effects. This suggests that animals with more receptor binding are less stressed compared to those that have less receptor binding. Adults of high LG-ABN mothers had significantly more binding of ligand to the $GABA_A$ receptor (Caldji et al., 2000). Levels of $\alpha1$ and $\gamma2$mRNA, involved in the functional benzodiazepine-binding site described above, are also altered by maternal care (Caldji et al., 2000). Adult rodents of high licking and grooming mothers, have increased levels of $\alpha1$ and $\gamma2$mRNA in the amygdala and the locus coeruleus within the brain (Caldji et al., 2000). High LG-ABN mothers may produce offspring with altered mRNA levels in the amygdala, ultimately affecting the subunits of the GABA receptor and therefore producing offspring presenting with less anxious behaviour as receptor binding is higher. In addition to this, benzodiazepine receptor density is higher in offspring of high LG-ABN litters (Caldji et al., 1998), allowing for more binding to occur therefore rendering the pup less anxious for a longer period of time. Moreover, an increase in $\alpha2$ adrenoreceptor number is increased in offspring of high LG-ABN litters (Caldji et al., 1998). This receptor mediates synaptic transmission of nerve terminals and therefore alters noradrenaline production, reducing the physiological changes (e.g. heart rate increase) associated with stress (Park et al., 2013).

2.4.1.3 Production of stress hormones

It has been suggested that oxytocin has anti-anxiety effects and therefore promotes maternal care, as opposed to corticotropin-releasing hormone (CRH) which promotes anxiety and disrupts maternal behaviour (Uvnas-Moberg, 1997). Variations exist in the oxytocin receptor levels between low and high LG-ABN mothers (Francis et al., 2000). There are also sex differences in oxytocin and vasopressin (hormones associated with anxiety and social behaviours). Adult females of high LG-ABN mothers exhibited increased oxytocin receptor binding, which was not seen in the adult males from the same cohort. In contrast, the adult males of high LG-ABN mothers had an increase in vasopressin receptor binding in the amygdala, while no difference was seen in the females (Francis et al., 2002). An increase in vasopressin receptor binding suggests that the individual is less likely to produce a large quantity of cortisol, as the vasopressin receptor is also related to adrenocorticotropic hormone (ACTH) release. Moreover, the impact of maternal care on adult offspring behaviour may be dependent on the sex of the offspring. This may be due to differences in developmental stages. The amount of licking in rat litters did not differ due to the sex of the individual (Francis et al., 2002).

2.4.1.4 Spatial learning and memory

Higher levels of anxiety are likely to impair learning. Differences in the latency and swim path of rodents in the Morris water maze test, across observation days, have been documented in litters with differing maternal care. The Morris water maze test is the gold standard measure of spatial learning and memory in rodents (Milner et al., 2014). There was significantly more searching in the target quadrant in adult rodents of high LG-ABN mothers compared to low LG-ABN mothers (Liu et al., 2000). Offspring of high LG-ABN mothers also showed a significantly shorter latency and swim path to the target platform compared to adults of low LG-ABN mothers (Liu et al., 2000). This is likely due to differences observed in hippocampal development and in particular the expression of genes encoding for N-methyl-D-aspartate (NMDA) receptor subunits, important for spatial learning and memory (Liu et al., 2000). It is therefore probable that offspring of low LG-ABN mothers will have altered memory and learning capacity as they have lowered expression of genes encoding for NMDA receptor subunits (Morris et al., 1982; Milner et al., 1998; Whishaw, 1998; Wood et al., 1999).

2.4.1.5 Behavioural responses in offspring

The impact of maternal care can also alter behaviour. Offspring of low LG-ABN mothers have a decrease in benzodiazepine and α2 adrenoreceptor density (receptors involved in stress hormone production) along with an increase in corticotropin releasing hormone (CRH) receptor density in the locus coeruleus (Caldji et al., 1998). In a novel environment, this resulted in an increased startle response, a decrease in open-field exploration and a longer latency to eat food provided (Caldji et al., 1998). This suggests there are strong physiological and developmental links between maternal care behaviour and future offspring behaviour in rodents. Forced separation between the mother and the offspring also trigger behavioural changes and are now considered.

2.4.2 Experimental manipulation of the mother/progeny interaction and subsequent effects on offspring development and behaviour

Differences in the amount of time the mother spends away from the offspring may affect stress development in young. Mother departure from the litter, including separation (3-12 hours) and deprivation (≤24 hours) between postnatal days 0-14 can alter stress responses of offspring later in life (Plotsky and Meaney, 1993, Meaney et al., 1996, Lehmann et al., 1999 and Lippmann et al., 2007). Short separation of the pup and mother is followed by an increased amount of pup licking when they are reunited (Lee and Williams, 1974; Lee and Williams, 1976; Anisman et al., 1998). Benefits of additional licking include an increase in brain plasticity (plasticity allows the individual to change their body’s physiology and behaviour in different circumstances) and lower anxiety compared to those pups which were not removed (Liu et al., 1997; Liu et al., 2000). Interestingly, separating the mother and puppy for a longer period of time (three hours) may have detrimental effects on the offspring. Offspring randomly separated from the mother for 3 hours between 0800 and 1600 hours displayed an increased response to stress, increased anxiety (as observed by locomotor activity, rearing, grooming and startle response), and cognitive impairment (increased adrenocorticotropin and corticosterone concentrations) (Lippmann et al., 2007). Periods of deprivation between the mother and pup are also associated with decreased exploration, behavioural inhibition (avoidance behaviour), increased CRH mRNA in the paraventricular nucleus, decreased levels of GR mRNA in the hippocampus and an increase in corticosterone response to stress (this was determined when the pups were undertaking the open field and elevated plus-maze, conditioned freezing, active avoidance and water maze) (Plotsky and Meaney 1993; Meaney et al., 1996; Lehmann et al., 1999). Female pups separated from the mother for 5 hours a day pre-weaning displayed a reduced amount of maternal licking and grooming with their own litter (Fleming et al., 2002).

Artificial and maternal rearing of rat pups has been compared. Pups in artificial rearing (AR) groups were stimulated with a soft wet paintbrush over the anogenital region. For the minimum AR group, each pup was swabbed in the anogenital region twice a day for 30 seconds. In the maximum AR group each pup was swabbed in the anogenital region twice a day for 30 seconds and had overall body stroking eight times a day for 2 minutes beginning at postnatal day 3 or 4 until postnatal day 16 (Lovic et al., 2006). The AR groups were more active in response to novelty tests compared to the mother-reared groups (Lovic et al., 2006). In contrast to the outcome reported by Lovic et al. (2006), Gonzalez and Fleming (2002) determined that AR groups are more likely to react negatively during experiences compared to those pups reared by their mothers. Physiologically there was a significant reduction in c-fos immunoreactivity in the medial preoptic area and the parietal and piriform cortices, areas which are involved in stress and pain response. Methodological differences could explain the outcome of results observed between AR and naturally reared rodent litters. Lovic et al. (2006) and Gonzalez and Fleming (2002) both separated the pups from the entire litter, including the mother. However, when the pup remains with their littermates and the mother is separated, changes in pup locomotion were not observed (Matthews et al., 1996; Brake et al., 2004). This has identified that the separation duration along with the separation method (whether the pup remained within the litter or was alone) are important in determining the response the offspring will have later in life.

2.5 Conclusion

In this Chapter, the literature relating maternal care to future offspring behaviour was explored. Parallels were drawn from rat literature, where abundant information highlights a correlation between naturally occurring differences in maternal care, and physiological and behavioural responses in offspring. Differences in mothering ability may naturally exist in dogs; however, there is a lack of documented maternal care behaviour in dogs within the first two postnatal weeks. It is difficult to relate literature describing behaviour in domestic dams because methods differ widely. Knowledge of maternal care behaviour in domestic dogs has implications for breeding plans where a more appropriate dam may be selected, resulting in better-mothered puppies. However, there is a need to document maternal care behaviour in domestic dogs before determining whether a relationship exists between maternal behaviour and future offspring behaviour, as it does in the rat. In the next Chapter, maternal care in domestic dogs will be compared using different sampling methodologies.

Chapter 3. Sampling maternal care behaviour in domestic dogs

In Chapter 2 the importance of maternal care and its influence on future offspring behaviour, and methods used to quantify maternal care behaviour were defined. Acknowledging the way in which maternal behaviour is measured is important, as it may alter the findings. Therefore, in this Chapter several methodologies will be compared using the 24-hour maternal care period in an effort to determine the extent of the variation caused by sampling methods, as well as documenting the most accurate method to measure maternal care in dogs.

3.1 Introduction

The selection of time sampling method is critical for accurately describing the frequency and duration of behavioural traits (Altmann, 1974; Daigle and Siegford, 2014). Ideally, the measurement period should be continuous to ensure that every event is measured within that period. However, continuous monitoring over long periods of time is rarely practical, with researchers often opting to measure behaviour across intervals. It is important that the frequency and duration of the behaviour of interest be ascertained prior to the start of any study (Altmann, 1974), as different time periods are required to identify specific behaviours (Daigle and Siegford, 2014). That is, the relative magnitude of the sampling error will depend on the frequency of the behaviour, its duration, and the number of observations made over the sampling period (Powell et al., 1977; Saudargas and Zanolli, 1990). In particular, infrequent behaviours or those with short durations can be erroneously measured if a short time sampling method is used (Saudargas and Zanolli, 1990). The importance of time sampling methodology and its implications have been documented and compared in other species (e.g. laying hens: Daigle and Siegford, 2014; rodents: Saibaba et al., 1996), but not so in the domestic dog. Although the principle of, and consequences of sampling methods extend across most species, it is uncertain how the methodology used to date impacts our understanding of maternal care in dogs.

A number of maternal care behaviours in domestic dogs have been explored during the first few weeks after birth. The behaviours documented are specific to the duration, and at times the frequency, of dam presence, nursing, contact and anogenital licking (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a and 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016; Guardini et al., 2016 (Table 3)). However, it is difficult to compare the findings of these studies due to inconsistent behavioural definitions and measurements that have been used (Table 3). For instance, the way in which contact between dam and puppy has been measured varies across studies. Rheingold (1963) defined contact as any physical contact between the mother and puppies, while Foyer et al. (2016) recorded contact as the duration of time the mother has elbows on the ground and is in physical contact (excluding the tail and limbs) with at least one puppy within the whelping box. There are also differences in the methodologies employed for quantifying maternal behaviours. For example, anogenital licking has been measured both as a frequency (Korda and Brewinska, 1977a; Grant, 1987) and duration (Rheingold, 1963; Guardini et al., 2015; Foyer et al., 2016). As Table 4 demonstrates, the sampling methodologies used across various studies has resulted in variation in the reported duration of the behaviour. To date, studies focusing on maternal care only describe the dam when she is with the entire litter (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a and 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016; Guardini et al., 2016), which ignores the interactions between dam and individual puppies. Thus, our understanding of the frequency and duration of maternal care behaviours in dogs remains relatively uncertain.

Table 3: Comparison of studies describing maternal care behaviours (dam presence, nursing, contact and licking) in dogs within the first 12 postnatal days.

Study	Breed and dams observed	No. of puppies	Sampling method	Sampling time (day/night)	Sampling range (postnatal days)	Total observations (hrs)
Rheingold, 1963	Cocker Spaniel (1) Sheltie (3) Beagle Total = 5	24	Average of 4 x 15 minutes each day	0800-1800 hr (day)	1-68	153
Scott and Fuller, 1965	Basenji (4) Beagle (5) Cocker Spaniel (5) Sheltie (5) Fox Terrier (5) Total = 24	DNS	10 minute weekly observations	DNS* ('daily')	1-28	20
Korda and Brewinska, 1977a, 1977b	Control: Mongrel (4)	32-36	14 hour daily continuous sampling	DNS ('daily')	2-4 and 13-16	784-882
Grant, 1987	Beagle (1)	7	One hour per day across a 24-hour period, on days 1-4, 7-10, and 12-21	24 hours (day and night)	1-21	18

*DNS = Data not shown.

Table 3 continued

Study	Breed and dams observed	No. of puppies	Sampling method	Sampling time (day/night)	Sampling range (postnatal days)	Total observations (hours)
Guardini et al., 2015	Weimeraner (1) Belgian Shepherd Groenendael (3) Cross-bred (1) Short Haired Dachshund (1) German Shepherd (1) Labrador Retriever (1) Boxer (1) Border Collie (1) Total = 10	58	15-minute video recording	Morning (day)	1-21	52.5
Foyer et al., 2016	German Shepherd (22)	94	Every second hour (12 hours) on one day, once a week	24 hours (day and night)	1-21	1056
Guardini et al., 2016	Beagle (8)	54	15 minutes once a day, every day for the first three postnatal weeks	Morning (day)	1-21	5.25

Table 4: Comparison of studies reporting maternal care behaviour in dogs within the first twelve postnatal days. The range in the duration of a behaviour reported reflects differences between dams.

Behaviour observed	Behaviour duration (%)
Dam presence	20-100[a#]
	58-81[b]
	60-98[c#*]
Nursing	3-84[a#]
	13-83[d]
	82-99[e#]
	20-63[b#]
	0-100[f#]
	8-23[c#*]
Contact	10-100[a#]
	29-86[e#]
	10-32[c#*]
Licking	0.1-8.9[a#]
	14-61 acts[b#]
	0-100 acts[e#]
	0-33.9[f#]
	1-5[c#*]

[a]Rheingold, 1963, [b]Grant, 1987, [c]Foyer et al., 2016, [d]Scott and Fuller, 1965, [e]Korda and Brewinska, 1977a, 1977b, [f]Guardini et al., 2015
[#]Behaviours were determined from figures only (no information provided in text).
*Behaviours were averaged per litter size and therefore behaviours are presented per puppy.

In this Chapter, the aim was to determine whether time sampling methodology influences the reporting of maternal care behaviours in domestic dogs. This study focussed on maternal care exhibited during the first twelve postnatal days, and compared time sampling methods used in previous studies to the frequency and duration of behaviours averaged over three 24-hour periods. The rationale for limiting our analysis to the first two weeks post-natal, were two-fold: First, this is the time when the maternal behaviours of interest occur most frequently (Kendrick et al., 1997). Second, maternal care, and in particular anogenital licking, within the neonatal period is important due to the likely influence on offspring stress development and behaviour (as described in Chapter 2). With this in mind, an additional aim was to rank puppies according to the frequency in which they are licked (in the anogenital area) by the dam. Doing so will enable researchers to determine whether the rate that the puppies were licked remained consistent across all sampling methods.

3.2 Methods

3.2.1 Animal subjects

Data from six litters of domestic dogs were used in this study (see Table 5). For litters where puppies were different to distinguish, a unique collar (i.e. ribbon, paper wrist band) was placed around their necks. This was done with permission from the breeder, and appeared not to disrupt either the dam or the puppy. Breeders were asked not to alter their normal routine, and at no time did the researchers interact with the dam or puppies. Ethics approval was granted from the University of Adelaide Animal Ethics Committee (S-2014-098).

Table 5: Dam and litter details

Litter	Breed	Dam and litter location	Dam parity	Dam age (years)	Litter size (postnatal day 1)
1	English Staffordshire Terrier	Inside house	1	2.5	6
2	Whippet	Inside house	3	6.5	5
3	Greyhound 1	Room outside house	4	8	5
4	Greyhound 2	Room outside house	1	5	3
5	Labrador	Inside house	3	3	1
6	Border Terrier	Inside house	2	3.5	5

3.2.2 Classification of maternal care behaviours

Four maternal care behaviours, adapted from Rheingold (1963) and Zahed et al. (2008), were coded using behavioural analysis software (Mangold Interact, v.9). Behaviours included: *dam presence* (any time in which the dam had at least one paw within the whelping box); *Nursing* (any time in which a puppy had its mouth around a teat of the dam (Table 6). The dam could be sitting, lying on her side or standing); *Puppy in contact* *with another individual (dam or puppy)* (any time in which a puppy had at least 50% of its body touching the dam or a littermate. This included contact while sitting or lying); and *Anogenital licking* (any movement of the dam's tongue along the genitalia or anus of a puppy). Nursing and Puppy in contact with another individual (dam or puppy) were not mutually exclusive behaviours. Based on the frequency in which the behaviours have occurred in previous studies, Dam Presence, Nursing, and Puppy in contact with another individual (dam or puppy) were deemed 'frequently occurring behaviours', and Anogenital licking was considered an 'infrequently occurring behaviour.

Table 6: Ethogram and operational definitions of domestic dog maternal behaviours

Behaviour category	**Description**	**Photo of behaviours used within the study**
Anogenital lick	Movement of tongue along the genitalia or anus of a puppy to stimulate urination or defecation.	
Nursing	Puppy has its mouth around a teat of the dam. Often includes the puppy placing its paws around the teat while moving their head backwards and forwards. The dam can be standing, sitting or lying on her side.	
Puppy in contact with another individual (dam or puppy)	A puppy is in a lying or sitting position and has at least 50% of its body against or alongside another puppy or the dam.	

3.2.3 Time sampling methods

Litters were video-recorded continuously using a fixed surveillance camera (Swann PRO-530) mounted in a position that overlooked the whelping box. Video was recorded at 20 frames per second with 1024 maximum bitrate (Kbps) using a digital video recording device (4/8 Channel D1 Realtime H.264 DVR). Recording occurred from postnatal day 0 until postnatal day 13, except for Litter 4 which was recorded from postnatal days 5-13. Each of the litters were coded for 96 hours over postnatal days 3, 6, 9 and 12 (24 hours per day), with the exception of Litter 4, which was coded for 72 hours over postnatal days 6, 9 and 12.

The following sampling methods were used: *24-hour:* Each of the four postnatal days were coded continuously for the 24-hour period. The average of these four 24-hour periods were used as the 'gold standard' method to which the following time sampling methods were compared. *12-hour night* and *12-hour day*: The 24-hour periods were split into two 12-hour periods covering the night (1800-0600 h) and the day (0600-1800 h). *One-hour anytime (set 1 and 2)*: Four random 15-minute periods were selected from each 24-hour period using the 'Random Number Generator' mobile phone application (Skytrait, V 4.2.0). The four 15-minute periods were randomly selected for each of the one-hour sampling methods to ensure repeatability of the measure when a different random selection were chosen. *One-hour night (set 1 and 2)* and *One-hour day (set 1 and 2)*: Four random 15-minute periods were selected from the night (1800-0600 h), and during the day (0600-1800 h).

3.2.4 Time sampling method analysis

Anogenital licking was measured using frequency. The frequency data were standardised per hour to allow comparison between sampling methods. Dam presence, nursing, and puppy in contact with another individual (dam or puppy) behaviour were measured in time, and calculated as a percentage of the total time the dam was present in the whelping box to account for differences in recording times. Puppies were also ranked across days for each sampling method to allow differences in ranking to be described. The litters were ranked for each behaviour from highest to lowest across each observation day for anogenital licking only. Focus was placed on anogenital licking because of its likely importance on future stress behaviour in puppies (see Chapter 2), and to determine how sampling methodology influences the measurement of infrequently occurring behaviours.

Data for the four behaviours were analysed using a multivariate general linear model in SPSS (IBM, v.21) to determine whether there was a difference in frequency or duration between the time sampling methods. Normality tests determined all behaviours (dam presence, nursing, contact and anogenital licking) were not normally distributed and therefore the data were log transformed. Post hoc tests, using Least Square Differences, were used to determine significance between time sampling methods. The Wilcoxon Signed Ranks Test was used to determine differences in puppy ranking in regards to anogenital licking between 24-hour, 12-hour night and 12-hour day across the four coding days. Significance was accepted at $p<0.05$.

3.3 Results

3.3.1 Frequently occurring behaviours

3.3.1.1 Dam presence

The amount of time the dam spent within the whelping box significantly differed between the 24-hour period and the time sampling methods (F (8, 113) = 2.50, p = 0.015; partial η^2 = 0.15). The 12-hour day time sampling method resulted in a significant reduction in dam presence compared to the 24-hour period (Figure 2.A). There was no significant difference between the one-hour periods when comparing random set 1 and random set 2 for the night, day, or anytime period ($p>0.05$).

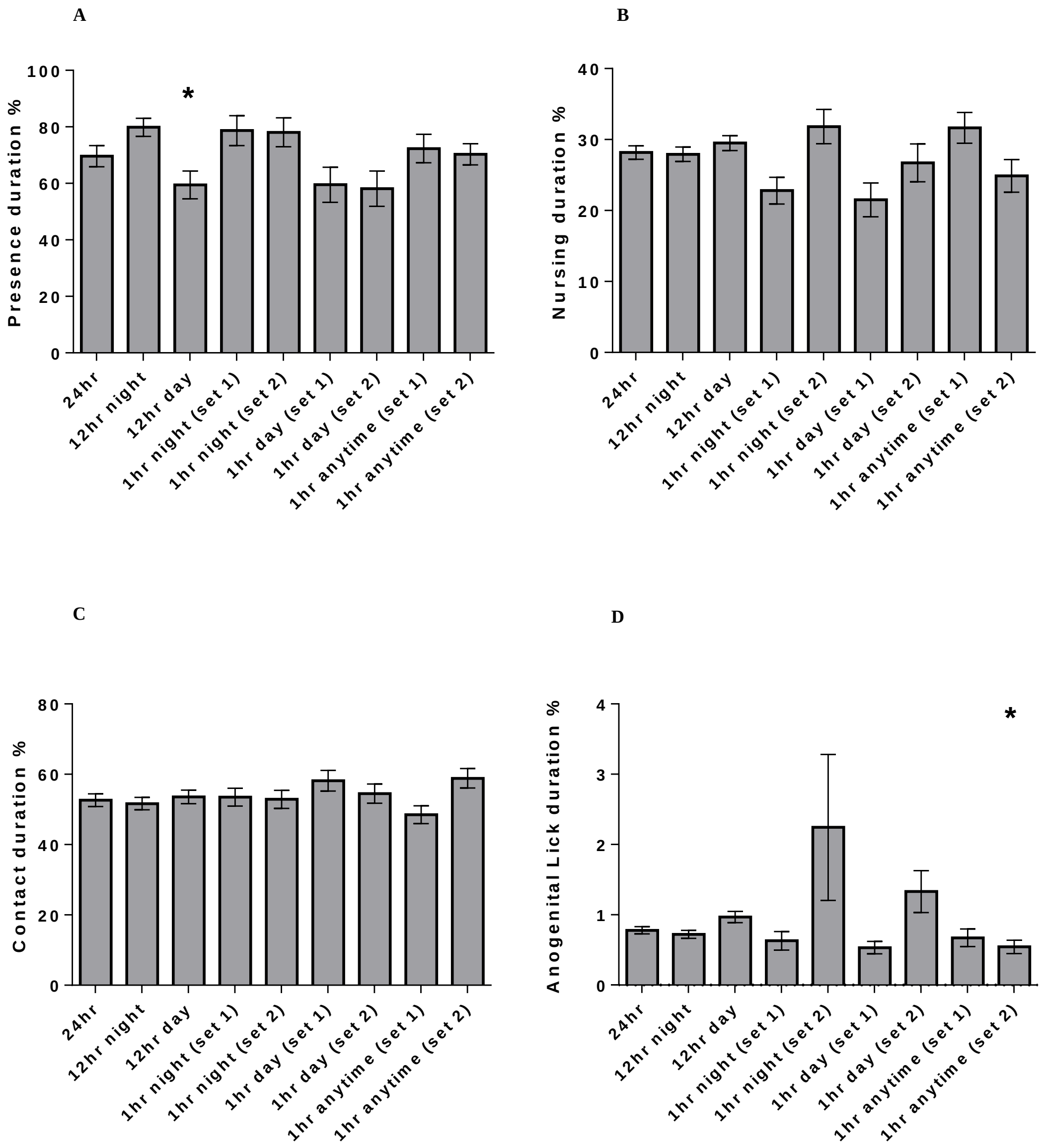


Figure 2: Comparison of time sampling methods for A) Dam Presence, B) Nursing C) Puppy in contact with another individual (dam or puppy) and D) Anogenital Licking. Values are expressed as a mean value of the four coding day's ± standard error of the mean. * indicates a significant difference to the 24-hour period when comparing log transformed data.

3.3.1.2 Nursing

There were no significant differences (F (8, 113) = 0.82, p = 0.585; partial η^2 = 0.06) between the 24-hour period and any of the time sampling methods (Figure 2.B). Both one-hour random set 1 and random set 2 (for night, day and anytime periods), did not significantly differ to one another (p>0.05).

3.3.1.3 Puppy in contact with another individual (dam or puppy)

There were no significant difference (F (8, 113) = 1.46, p = 0.179; partial η^2 = 0.09) between the 24-hour period and any of the time sampling methods for contact behaviour (Figure 2.C). No significant differences were found between the random set 1 and random set 2 for the short time sampling methods (one hour night, day and anytime periods).

3.3.2 Infrequently occurring behaviour

3.3.2.1 Anogenital Licking

Differences were observed between the 24-hour period and time sampling method (F (8, 113) = 2.04, p = 0.048; partial η^2 = 0.13). The one hour anytime (set 2) time sampling period showed a significantly higher amount of anogenital licking compared to the 24-hour period (Figure 2.D). Although there was a significant difference between one-hour anytime random set 1 and 2, there were no significant differences between one-hour random set 1 and set 2, or between the one-hour night and one-hour day periods (p>0.05). In regards to anogenital licking frequency, both random set 1 and set 2 for the one hour time sampling methods (night, day and anytime) significantly underestimated anogenital licking compared to the 24-hour period (Figure 3).

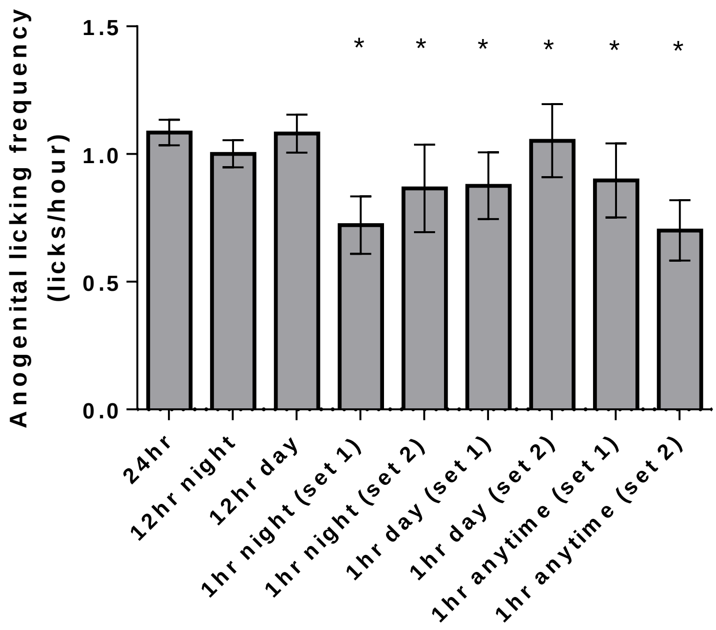


Figure 3: Comparison of different time sampling methods for frequency of anogenital licking per hour. Values are expressed as a mean of the four coding day's ± standard error of the mean. An asterisk marked above a sampling period indicates a significant difference to the 24-hour period when the data were log transformed.

There were no significant differences in the ranking results when the 12-hour night (Z=-0.339, p=0.735) and the 12-hour day (Z=-0.222, p=0.824) time sampling methods were compared to the 24-hour period (p>0.05). However, all six variations of the short time sampling methods (one hour) were significantly different to the 24-hour period (p<0.05). This is discussed further in Chapter 4 of this thesis.

3.4 Discussion

The aim of the study in this Chapter was to determine which time sampling method was optimal for measuring the frequency and duration of maternal care behaviour in domestic dogs. To achieve this, the behaviours were compared using a variety of time sampling methods to a 24-hour period. It was determined that the accuracy of the observations related to the time sampling method used. That is, longer time sampling methods (e.g.

12-hour day and 12-hour night) can be used in lieu of a 24-hour period for some frequently occurring behaviour such as nursing and contact behaviour, and a 12-hour night time sampling period representative of dam presence. In fact, a time sampling method as short as one-hour (consisting of four 15-minute periods, randomly selected during the day, night or anytime throughout the 24-hour period), may be used for some frequently occurring behaviours across a 24-hour period.

For infrequent behaviours such as anogenital licking however, the use of short time sampling methods do not accurately reflect the frequency or duration when compared with a 24-hour period. Further, when short time sampling methods were used to measure anogenital licking in the current study, the results were highly inconsistent. This finding is not unexpected, as several studies have reported that short time sampling methods do not accurately describe infrequent and low duration behaviours (Powell et al., 1977; Saudargas and Zanolli, 1990). Rheingold (1963) and Guardini et al. (2015) have previously explored anogenital licking, but given their sampling methodology, it is likely that their results do not accurately represent the true behaviour. Accurately describing the frequency of behaviours is critical, as for example, the amount of anogenital licking a puppy receives may in fact alter its brain development and their response to stressful situations later in life (see Czerwinski et al., 2016).

In observational studies of maternal care behaviour in dogs, it is common for the behaviours to be recorded for the entire litter and hence coded from the perspective of the dam. This presents the opportunity to gain a true picture of the behaviour of the whole litter, but prevents the ability to detect individual differences in puppy behaviour, or the interaction between the dam and puppies. For example, when coding at the litter (or group) level, it is impossible to distinguish how many, or which, individual puppies are feeding. Within the current dataset, puppies were individually identified, and their behaviours recorded (with the exception of dam presence). We found that dams engaged in anogenital licking between 0.09-4.10% of the time when observed for a 12-hour period. This was similar to the range (1-5%) observed by Foyer et al. (2016). However, in that study, the duration of anogenital licking was estimated across litter size and they were not able to distinguish individual puppies consistently. It is highly likely that dams do not provide equal care among all her offspring, and thus, the development of each puppy might differ as a result.

Within the current study, we found no difference between the 24-hour period and the 12-hour periods for the frequency of anogenital licking (either across the night or day), suggesting that 12 continuous hours coded either day or night, is representative of the behaviour across a 24 hour period. However, it is likely that the definition of anogenital licking is more influential than the sampling method. For example, Korda and Brewinska (1977a) documented between 30 and 100 licking acts per litter within the first twelve days postnatal, while we reported a much smaller range (0-41 licks per litter). In our study, each licking event was recorded regardless of the duration in which it occurred, whereas Korda and Brewinska (1977a) recorded anogenital licking events with a duration over 30 seconds, and as a result, one licking event could include multiple puppies.

To further understand sampling methodology and its accuracy, it is important to determine the number of hours over which an infrequent behaviour can be determined. It may be possible that a time period anywhere from one to 12 hours can provide representative results. To gain a more accurate picture of infrequent behaviours, a more selective period may improve the likelihood of capturing the behaviour. Guardini et al. (2016) for example, coded behaviour for 15 minutes after the dam had returned to the litter in the morning. This is likely to capture a high level of activity, given the separation period of the mother from the litter can stimulate a greater expression of maternal behaviour when the dam returns. Further, the dogs in our study represented a number of breeds, dams differed in relation to maternal experience (parity), and the size of each litter was variable. The true impacts of each of these aspects are currently uncertain, and should be investigated further. Lastly, our data came from dogs living in the breeder's household (which comes with potentially distracting human daytime activity), where some of the previous studies reporting less variance were measured in presumably more constant laboratory situations. Thus, we also recommend that future studies observe the interaction of the handler/breeder on the litter to determine the impact they may have on the dam's behaviour.

3.5 Conclusion

In this study, evidence was found to suggest that not all behaviours need to be observed for a full 24-hour period to get an adequate representation of frequency or duration. Short time sampling methods can accurately represent a 24-hour period, but only for frequently occurring behaviours. These include: dam presence, nursing, and lying in contact with another individual (dam or puppy). For the assessment of low-frequency behaviours, such as anogenital licking, short time sampling methods (e.g. one-hour duration), are unsuitable and in these situations, a longer, continuous period of time of observation is recommended. These findings highlight the influence of time sampling on behaviour representations, and provide useful insight for future studies measuring maternal care in canids, as well as in other species.

Chapter 4. Maternal care behaviour of domestic dog bitches

In the previous Chapter, maternal care behaviour was assessed using different coding methodologies. A short time sampling method for frequent behaviour and a longer time sampling method for infrequent behaviour were considered representative of a 24-hour period. These methods were used to observe and record maternal care behaviour in domestic dog litters as a prelude to determining within and between litter differences on subsequent stress responses of offspring.

4.1 Introduction

Parental care is considered to greatly influence offspring behaviour, especially maternal care. Numerous studies investigating maternal care and separation in rhesus monkeys documented behavioural changes when young are removed (Seay et al., 1962; Seay and Harlow, 1965; Hinde and Spencer-Booth, 1971). When the infant is separated from the mother, the infant immediately increases vocalisation and displays agitated locomotor behaviour, and after several days infant activity and play duration decreases (Seay and Harlow, 1965). Offspring reared by mothers, as opposed to pair- or group-reared infants, play more often and when adults they were less hostile and they interacted with others more (Chamove et al., 1973). Group- and pair-raised infants showed inhibited social development compared to those raised by a surrogate or natural mother (Chamove et al., 1973).

Maternal and paternal genetics may impact offspring behaviour. Intergenerational transmission of maternal care behaviour in rats has been documented, suggesting a genetic influence on maternal care behaviour (Caldji et al., 2000). Behaviour can also be impacted by environment, as events can alter gene expression (Caldji et al., 2000). Haplotypes regulating gene expression vary and are suggested to be transmitted across generations (Caldji et al., 2000). Different positions on several chromosomes are related to maternal care behaviour in rodents (Ashbrook et al., 2015). Such differences were observed during postnatal week one where nest building was compromised by a specific locus on offspring chromosome 7, suggesting a possible maternal-offspring gene interaction (Ashbrook et al., 2015).

Gene expression alters maternal care behaviour in rats (Patisaul et al., 2003; Champagne et al., 2006; Weaver et al., 2006; Ruthschilling et al., 2012). In the olfactory bulb of Wistar rats, high licking mothers had increased transcriptional expression levels of multiple receptors, namely serotonin (HTR1a, HTR1b), estrogen (Erα), dopamine (D1a) and prolactin (Prlr) (de Moura et al., 2015a). Histones, which regulate gene expression, display a distinct specificity which may be stimulus dependent (Bredy et al., 2007; Elsner et al., 2011; Lalonde et al., 2014). Modifications in histones have been associated with depression, schizophrenia and addiction (Kumar et al., 2005; Sharma, 2005; Tsankova et al., 2006) providing a potential link to differences in behaviour patterns. High licking mothers showed an increase in histone-H4 acetylation compared to low licking mothers, as reported by de Moura et al. (2015b). The effect of genetics on maternal care is still not completely understood, however environmental impact on maternal care is documented in altricial species, as discussed below.

Maternal care is particularly important in altricial species born physiologically immature and requiring care for development and survival (Kendrick et al., 1997). Care-giving behaviours in canids include feeding, regulating body temperature and facilitating urination and defecation (Walker, 2010). Maternal care encompasses both direct and indirect behaviours. Direct behaviours include those targeted toward offspring, such as licking, contact and nursing (Kendrick et al., 1997). These behaviours are particularly important in canids where the dam licks puppies to stimulate urination and defecation

(Brouette-Lahlou et al., 1992) and maintains contact with puppies for thermoregulation. In domestic dogs, the neonatal period ranges from birth to two weeks of age. The puppy is completely dependent on the mother for protection, warmth and food as limited vision, hearing and motor functions make puppies vulnerable in this period (Freedman et al., 1961; Scott and Fuller, 1965). Indirect maternal behaviours include those required to protect puppies and the dam may become aggressive towards intruders (Kendrick et al., 1997).

Although early developmental behaviour of canid species is well understood (e.g. Menzel and Menzel, 1936; Freedman et al., 1961; Scott and Fuller, 1965; Caro and Bateson, 1986; Serpell and Jagoe, 1995; Malm and Jensen, 1997), maternal care behaviour in domestic dogs is less studied. Maternal care can alter puppy development and affect the nervous system (Chapillon et al., 2002) and HPA axis development (Wigger and Neumann, 1999; Slabbert and Rasa, 1993). The most critical time to observe maternal care and its impact on the HPA axis is within the first 10 days postnatal (Levine and Lewis, 1959; Meaney and Aitken, 1985; Myers et al., 1989). Within this period the HPA axis is predominantly affected by the interaction between mother and puppy (Kalinichev et al., 2002). Anogenital licking is undertaken to elicit urination and defecation, and licking duration can alter HPA axis activity and brain development, as observed in rats (Greenough, 1990; Lenz and Sengelaub, 2009). Naturally occurring differences in maternal care behaviours, such as licking and grooming, have been observed within and between different breeds of rat (Champagne et al., 2003). These differences have profound effects on the offspring's behavioural and neural phenotypes (Pan et al., 2014). Offspring licked less showed an increased fear response to novelty (Caldji et al., 1998). These results are based on dam behaviour toward the litter, while within litter differences may also exist. Maternal care and future offspring behaviour has been thoroughly studied in rat, yet is limited in the domestic dog. Maternal care in domestic dogs may also play a vital role in puppy response to stress later in life.

Maternal care in puppyhood may significantly impact anxiety development in adult dogs, as shown by Tiira and Lohi (2015) (see also Chapter 1.1). However, insufficiencies in that study may have affected results as the respondent was not the breeder, but rather the dog's owner, who may not have known the mothering style of their puppy's mother possibly leading to a subjective answer. Nonetheless, these results are the first to indicate a correlation between maternal care and offspring behaviour later in life in domestic dogs. Direct evidence of a link between maternal behaviour and puppy anxiety later in life has been recently documented (Foyer et al., 2016). Maternal behaviour of 22 German Shepherd litters were observed for 12 hours a day, once a week during the first three postnatal weeks. The dams differed in the level of maternal care, in particular for duration of dam presence, nursing, contact, licking, and sniff/poke behaviour, and were separated according to whether they gave a low or high level of care. At 18 months of age, puppy behaviour was significantly affected (p<0.001) for physical engagement, social engagement and aggression. A combined maternal care score of contact, licking, anogenital licking and nursing was associated with less stress behaviour portrayed by the puppy at approximately eight weeks of age in an isolation test (Guardini et al., 2016). However, in the study conducted by Guardini et al. (2016) maternal care in the first three postnatal weeks was not described and a combined maternal care score, rather than individual maternal care behaviours, was used to determine the impact on puppy behaviour later in life. To more thoroughly investigate maternal care behaviour, dam behaviour may need to be observed more often during the first three postnatal weeks.

The implications of gaining insight into maternal behaviour in domestic dogs includes successful breeding programs. Dam selection is predominantly undertaken in regards to particular physical characteristics and temperament (King et al., 2009), yet maternal care may influence puppy behaviour but does not seem to be considered. To date, there is little documented information on maternal care behaviour in dogs within the first two postnatal weeks, as discussed in Chapter 2. Maternal care in domestic dogs is not well understood and remains relatively novel as a predisposing factor for dog stress and anxiety later in life. The aim of the current study therefore was to firstly determine whether puppies within a litter experience different levels of maternal care behaviours, and secondly to determine whether there are differences in maternal giving behaviours between domestic breeding bitches. This study helps determine whether differences in maternal care behaviour influence the stress response of puppies later in life. The following maternal care behaviours were observed and described: nursing, contact and anogenital licking.

4.2 Materials and Methods

4.2.1 Subjects

A total of 10 litters consisting of 10 bitches and 49 puppies participated in this study (see Table 7). Six different breeds were observed and dam age ranged from 1.8 to 8 years. Dam parity ranged from one to four. Both natural and caesarean births were recorded for the 10 dams and litter sizes ranged between one and eight. The dam and litters were filmed at the breeder's residence, all located in South Australia, Australia (litter 1: October 2014; litter 2: November 2014; litter 3: December 2014; litters 4 and 9: January 2015, litters 5 and 6: February 2014, litters 7 and 10: April 2015, and litter 8: May 2015). All 10 dog breeders had experience with dogs previously, however, only seven breeders had previous experience with whelping bitches. Breeders were recruited by social media and by directly contacting local registered dog breeders. Approval from the University of Adelaide Animal Ethics Committee was obtained (S-2014-098).

Table 7: Details of litters and dams (age in years, dam parity) used to describe maternal behaviour

Litter	Breed	Age (yrs.)	Dam Parity	Birth type	Litter size at birth	Litter size at postnatal day 3	Sex ratio (M:F) at postnatal day 3	Birth weight range (g)
1	English Staffordshire Terrier	2.5	1	Natural	7	6	3:3	184-245
2	Whippet	6.5	3	Natural	6	5	3:2	257-310
3	Greyhound 1	8	4	Caesarean	6	5	5:0	Did not measure
4	Greyhound 2	5	1	Caesarean	4	3	1:2	450-720
5	Labrador 1	3	3	Natural	1	1	0:1	696
6	Border Terrier	3.5	2	Caesarean	5	5	2:3	218-262
7	Labrador 2	2.2	1	Natural	7	7	3:4	593-640
8	Border Collie	4	1	Caesarean	6	6	4:2	270-420
9	Labrador 3	1.8	1	Natural	8	8	5:3	440-500
10	Labrador 4	4.2	3	Natural	3	3	1:2	434-562

4.2.2 Procedure

Each litter was filmed continuously for a period of 12 days, beginning on the day puppies were born. Location of the whelping box was determined by the breeder (litters 1, 2 and 5-10 were located within the house; litters 3 and 4 located in an enclosed room outside of the main residence). Prior to the expected whelping date, a surveillance camera was positioned above the whelping box to ensure coverage of the entire box, while not interfering with the dam, puppies or breeder (Figure 4). A camera (Swann PRO-530) was connected to a digital video recording device (4/8 Channel D1 Realtime H.264 DVR) and monitor. Video footage was recorded at 20 frames per second with 1024 maximum bitrate (Kbps). In order to identify puppies, a collar (a piece of fabric or paper wrist band that differed in colour, pattern and/or width) was tied loosely around each puppy's neck or the puppy was marked with nail polish on different sections of the body (head, neck, middle of back, lower back and tail). Collars or markings were placed on puppies by the breeder only when there were no identifiable features between puppies in the same litter. To allow for observation of naturally occurring behaviours, breeders were asked not to alter their normal routine, and the researchers did not interact with the dam or puppies at any time. A temperature thermometer (HOBO Onset Pendant Temperature Data Logger) was mounted and secured next to the camera to determine ambient temperature across the observation period. Ambient temperature was set to record every five minutes. Daily average temperature data for the observation days are presented in Table 8.

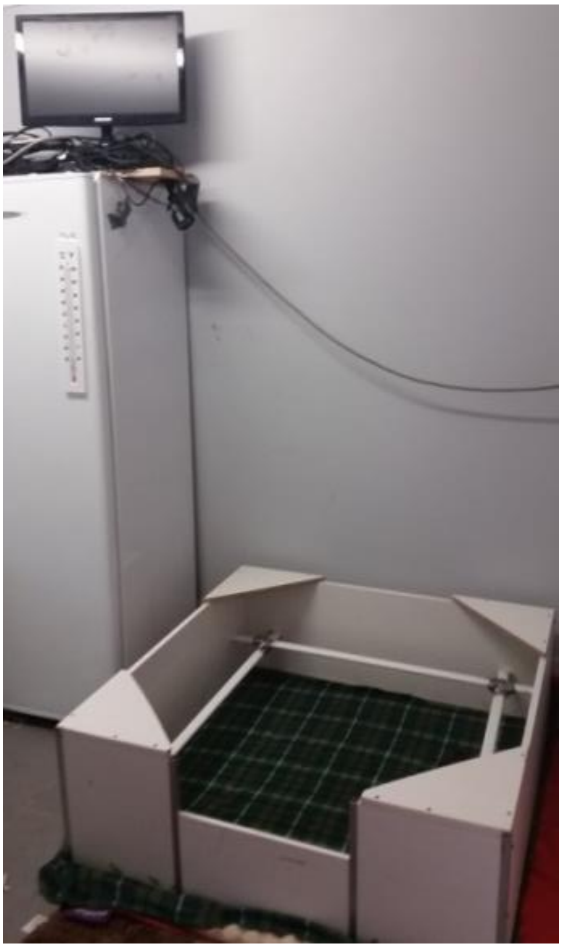


Figure 4: Set up of camera and equipment located above the whelping box.

Table 8: Average daily ambient temperature (˚C) for 10 litters across postnatal days 3, 6, 9 and 12.

Litter	Breed	Observation day	Average daily temperature (˚C)	Daily temperature (ºC) range
1	English Staffordshire Terrier	3	25.5	20.7-30.8
		6	24.5	22.5-27.3
		9	27.0	24.9-29.6
		12	26.3	24.7-27.7
2	Whippet	3	25.9	23.4-27.5
		6	24.1	19.9-27.3
		9	20.9	16.5-24.9
		12	26.1	22.2-31.8
3	Greyhound 1	3	22.5	14.1-30.6
		6	21.5	17.4-25.3
		9	18.9	13.3-24.6
		12	21.0	16.0-26.1
4	Greyhound 2	6	25.0	23.1-27.4
		9	23.6	19.5-28.7
		12	25.6	20.2-32.8
5	Labrador 1	3	22.7	20.1-25.6
		6	22.3	20.3-25.1
		9	23.3	21.4-26.1
		12	22.1	18.7-25.9
6	Border Terrier	3	22.4	20.2-24.4
		6	24.6	22.0-27.1
		9	23.3	21.1-25.3
		12	22.6	21.0-24.0
7	Labrador 2	3	27.6	26.2-29.1
		6	26.9	25.4-28.3
		9	26.8	25.8-27.6
8	Border Collie	3	19.0	18.1-20.3
		6	19.9	19.0-21.6
		9	20.0	19.1-22.1
		12	20.0	19.0-21.8
9	Labrador 3	N/A*	N/A	N/A
10	Labrador 4	6	29.0	27.9-30.4
		9	27.7	27.0-28.4
		12	26.4	25.7-27.3

*N/A = not available (temperature was not measured).

An ethogram (Table 6) relating to maternal behaviour was adopted from previous studies of dog development conducted by Rheingold (1963) and Zahed et al. (2008). Several behaviours were excluded from analysis as they either rarely occurred or did not occur (these included body lick and face lick), or behaviours did not relate to dam behaviour (such as puppy lying alone). To enable comparison between litters, nursing and puppy lying with another individual behaviours were expressed as a percentage of time the dam was present in the whelping box, while anogenital licking was expressed as an absolute value.

Observations of each litter were coded using methodology described as most accurate in Chapter 3. Two periods were used for coding: a one hour period made up of four randomly selected 15-minute periods across the whole day for frequent behaviours (puppy lying with another individual and nursing) and 12 hours of continuous observation during the day for infrequent behaviours (anogenital licking). Seven litters were observed across postnatal days 3, 6, 9 and 12, while the remaining three litters were observed on postnatal days 3, 6 and 9 or 6, 9 and 12 (Table 9). Total observation of maternal care behaviours for the 10 litters were 481 hours. Behaviours were coded using Mangold INTERACT software (v.9).

Table 9: Total observations of observed domestic dog litters for maternal care behaviour

Litter	**Breed**	**Observation day**				**Total hours of**
		3	**6**	**9**	**12**	**observation**
1	English Staffordshire Terrier	✓	✓	✓	✓	52
2	Whippet	✓	✓	✓	✓	52
3	Greyhound 1	✓	✓	✓	✓	52
4	Greyhound 2		✓	✓	✓	39
5	Labrador 1	✓	✓	✓	✓	52
6	Border Terrier	✓	✓	✓	✓	52
7	Labrador 2	✓	✓	✓		39
8	Border Collie	✓	✓	✓	✓	52
9	Labrador 3	✓	✓	✓	✓	52
10	Labrador 4		✓	✓	✓	39
						481

4.2.3 Statistical analysis

Data for puppy lying with another individual, nursing and anogenital licking were analysed using a multivariate general linear model with SPSS (IBM v.21) to determine whether there was a difference between and within litters across the four observation days. Shapiro-Wilk test was used to determine normality and found all behaviours (nursing, contact and anogenital licking) were not normally distributed and hence all data were log transformed before analysis. Post-hoc tests, using Least Square Differences, were used to determine significance between fixed variables (dam, day and puppy). Significance was accepted at $p<0.05$.

4.3 Results

4.3.1 Nursing

Nursing duration differences were observed across days and between litters (Table 10). Nursing was not observed in a number of litters across certain observation days, specifically the Labrador 2 and Border Collie litters on observation day 9 and Greyhound 2 litter on day 12. The largest range of nursing was observed in the Border Terrier litter on postnatal day 6, of 6.1 to 45.0 minutes (a difference of over 38.9 minutes).

Table 10: Nursing duration across postnatal days 3, 6, 9 and 12 for 10 litters

Dam ID	Breed	Day	Puppy duration range (m)	Total per litter (m)
1	English Staffordshire Terrier	3	0-19.6	73.7
		6	11.9-19.8	96.9
		9	4.0-17.1	58.7
		12	16.5-33.6	143.2
2	Whippet	3	2.4-3.3	13.8
		6	4.4-23.2	71.1
		9	10.2-25.6	95.3
		12	2.9-11.0	33.8
3	Greyhound 1	3	22.2-35.3	139.4
		6	2.5-10.5	40.9
		9	8.3-11.9	53.6
		12	10.1-14.3	58.8
4	Greyhound 2	6	9.0-23.0	42.6
		9	0-1.5	1.5
		12	0.0	0.0
5	Labrador 1	3	12.2	12.2
		6	11.5	11.5
		9	30.0	30.0
		12	14.8	14.8
6	Border Terrier	3	0.4-8.9	14.9
		6	6.1-45.0	111.9
		9	11.7-24.3	82.6
		12	0.4-8.9	14.9
7	Labrador 2	3	1.9-12.1	23.0
		6	10.9-17.8	41.6
		9	0.0	0.0
8	Border Collie	3	19.3-38.9	160.7
		6	0.0-14.3	40.0
		9	0.0	0.0
		12	0.0-1.0	3.4
9	Labrador 3	3	6.2-15.4	76.8
		6	0.3-11.4	37.1
		9	12.2-15.9	103.7
		12	6.7-10.8	63.6
10	Labrador 4	6	0.0-15.0	15.0
		9	0.0-1.32	1.3
		12	0.0-0.8	3.1

The percentage of time the dam spent nursing was significantly different between dams ($p<0.001$). The Whippet dam nursed her puppies more, and this was significantly different to all dams except Greyhound 1, Labrador 1 and Labrador 2. The Labrador 4 dam had a significantly lower duration of nursing compared to all other dams. There was also a difference in duration of nursing across days ($p=0.024$), where more nursing was observed on days 3 and 9, as compared to days 6 and 12. There were no significant differences between observation days 9 and 12. No significant differences were found in duration of time the dam spent nursing individual puppies in the litter ($p=0.963$) (Figure 5a, 5b and 5c).

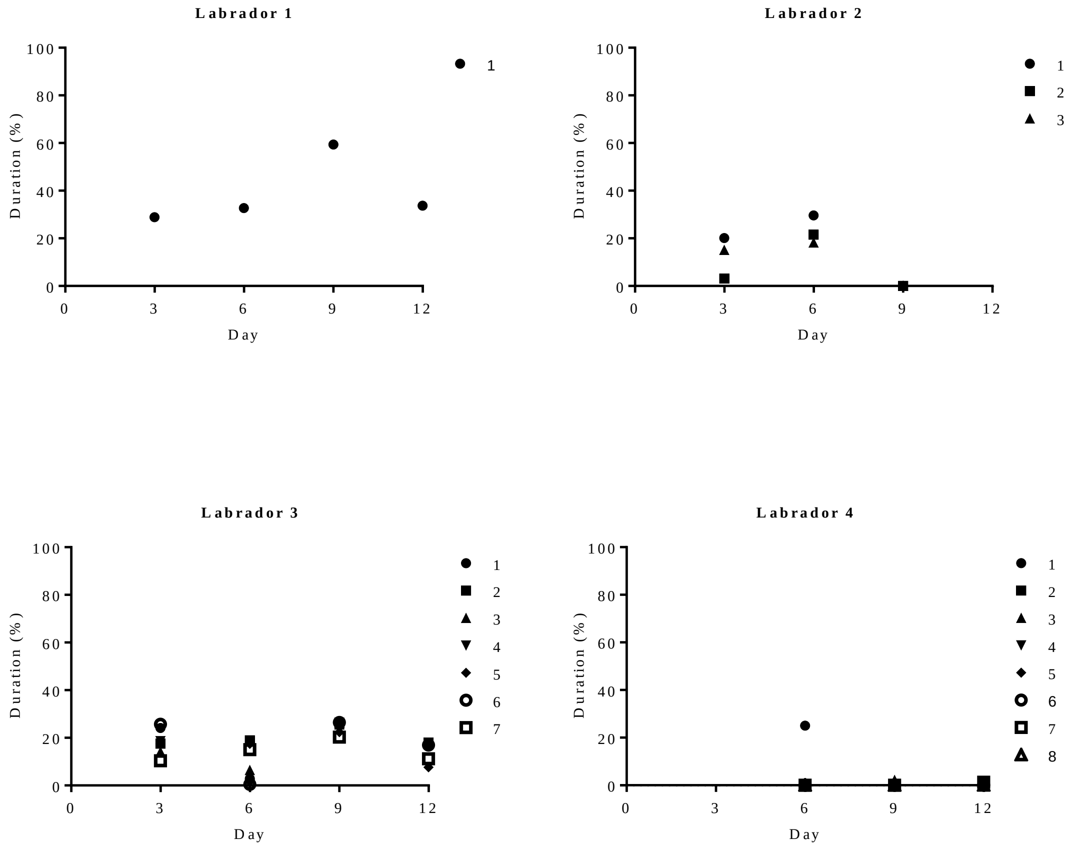


Figure 5a: Duration of nursing (%) per puppy on postnatal days 3, 6, 9 and 12 for Labradors. Results are separated per dam. Each puppy is represented by a different symbol.

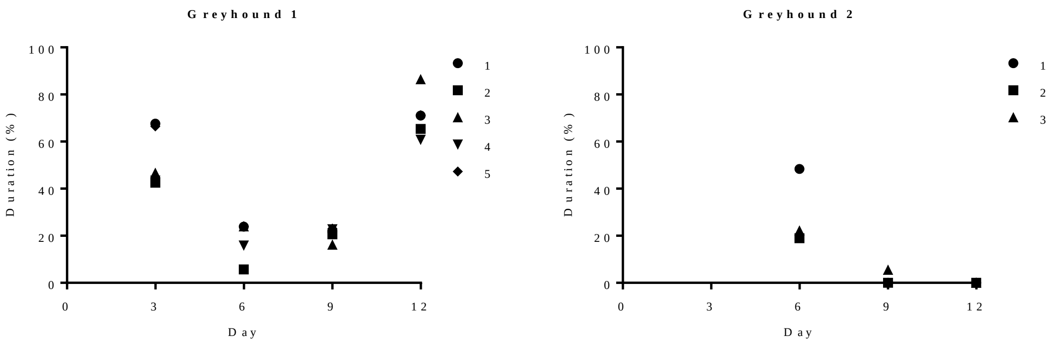


Figure 5b: Duration of nursing (%) per puppy on postnatal days 3, 6, 9 and 12 for Greyhounds. Results are separated per dam. Each puppy is represented by a different symbol.

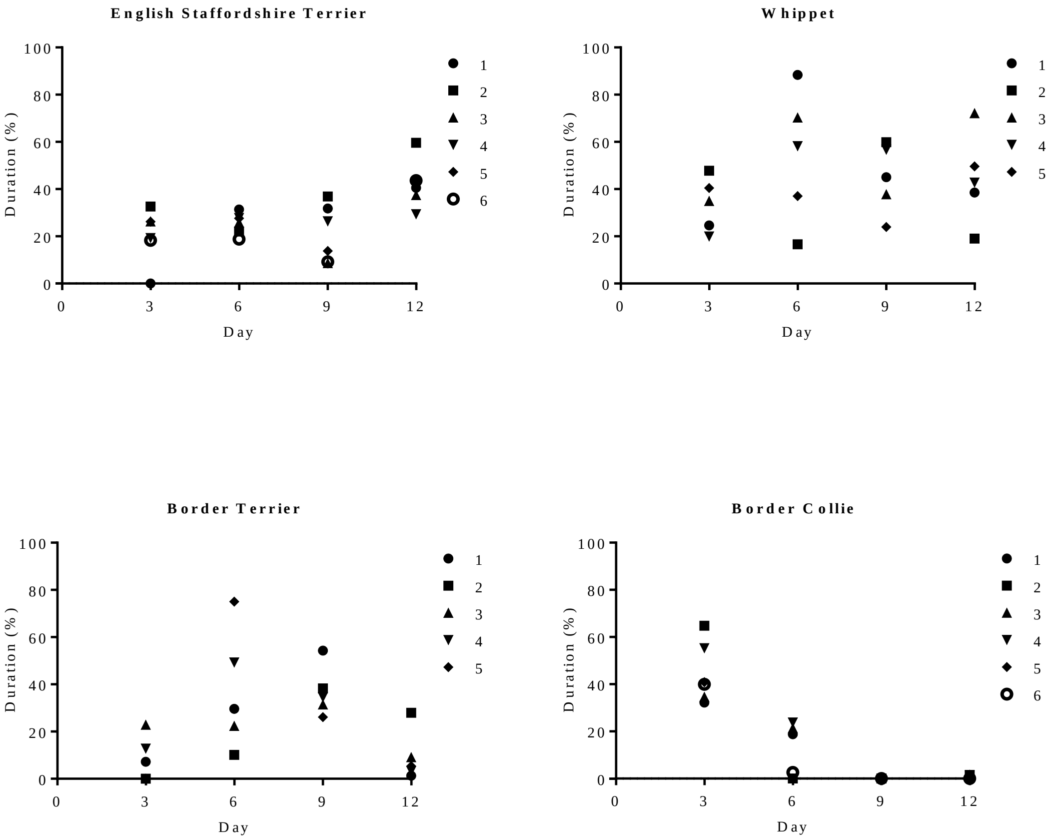


Figure 5c: Duration of nursing (%) per puppy on postnatal days 3, 6, 9 and 12. Results are separated per dam. Each puppy is represented by a different symbol.

4.3.2 Puppy lying with another individual (puppy or dam)

Differences in contact duration were observed between litters (Table 11). The Labrador 2 and Greyhound 2 litters were not in contact with another individual (puppy or dam) on days 9 and 12, respectively. The largest range in contact was observed in the Border Terrier litter on postnatal day 6, of 12.2 to 53.0 minutes (a difference of over 40 minutes).

Table 11: Contact duration across postnatal days 3, 6, 9 and 12 for 10 litters

Dam ID	Breed	Day	Puppy duration range (m)	Total per litter (m)
1	English Staffordshire Terrier	3	33.4-44.5	236.0
		6	21.3-55.5	267.2
		9	26.9-47.9	223.4
		12	6.4-28.3	120.4
2	Whippet	3	16.2-44.8	139.2
		6	6.1-39.0	108.6
		9	35.9-42.9	184.8
		12	12.1-28.7	90.4
3	Greyhound 1	3	8.2-14.2	52.1
		6	31.7-34.2	166.7
		9	36.2-43.3	196.2
		12	26.0-47.4	178.4
4	Greyhound 2	6	0.0-17.6	19.5
		9	1.6-4.6	6.2
		12	0.0	0.0
5	Labrador 1	3	18.9	18.9
		6	22.2	22.2
		9	0.9	0.9
		12	12.5	12.5
6	Border Terrier	3	25.4-41.5	164.1
		6	12.2-53.0	152.1
		9	17.2-45.2	165.2
		12	25.4-41.5	164.1
7	Labrador 2	3	0.0-11.7	15.2
		6	2.5-18.5	37.9
		9	0.0	0.0
8	Border Collie	3	4.3-21.1	52.0
		6	1.5-32.5	111.2
		9	42.5-57.4	289.5
		12	30.3-53.8	249.2
9	Labrador 3	3	13.7-37.6	165.8
		6	2.6-19.2	77.5
		9	0.0-5.8	16.2
		12	3.3-16.5	69.8
10	Labrador 4	6	3.4-22.8	113.7
		9	21.3-35.8	246.2
		12	8.7-30.0	152.3

There was a significant difference between litters in the amount of time the puppies spent lying by another individual ($p<0.001$). Puppies in the Border Terrier litter were in contact with another puppy or the dam more often, however this was not significantly different to Greyhound 1 and Labrador 1 litter. The shortest duration of contact was observed in the Greyhound 2 litter, but this was not significantly different to the English Staffordshire Terrier, Whippet, Labrador 2 or Labrador 3 litters. There were no significant differences in contact across observation days (p=0.062) or within a litter (p=0.584) (Figure 6a, 6b and 6c).

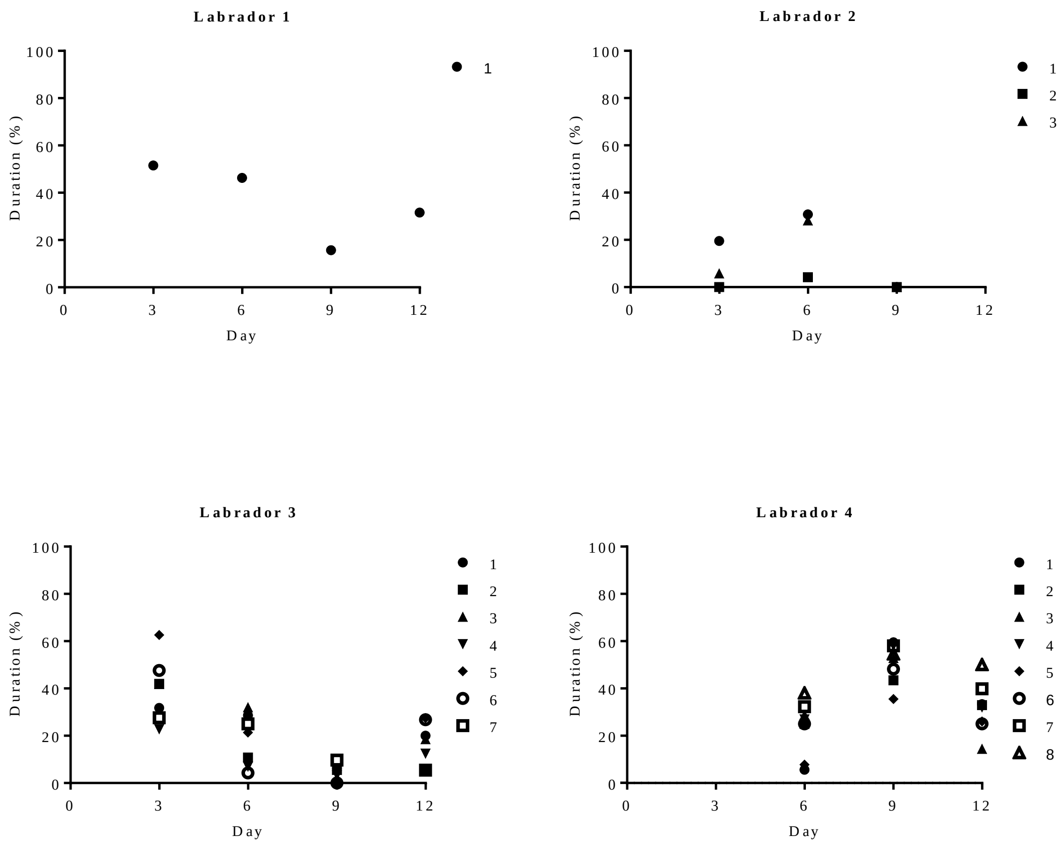


Figure 6a: Contact duration (%) per puppy on postnatal days 3, 6, 9 and 12 for Labradors. Results are separated per dam. Each puppy is represented by a different symbol.

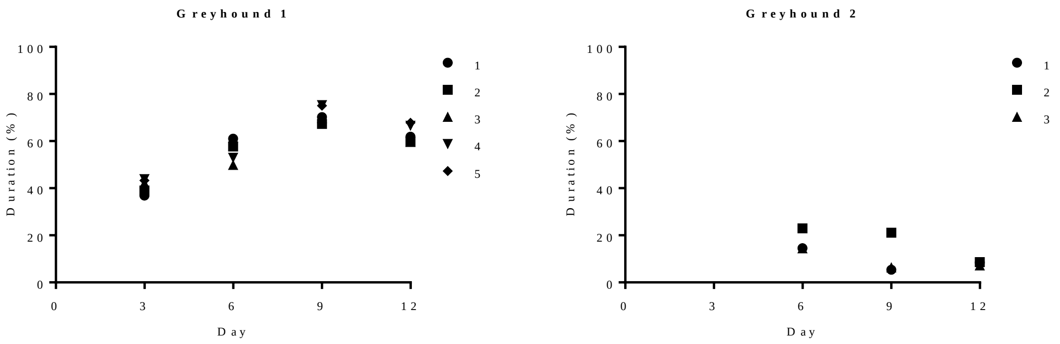


Figure 6b: Contact duration (%) per puppy on postnatal days 3, 6, 9 and 12 for Greyhounds. Results are separated per dam. Each puppy is represented by a different symbol.

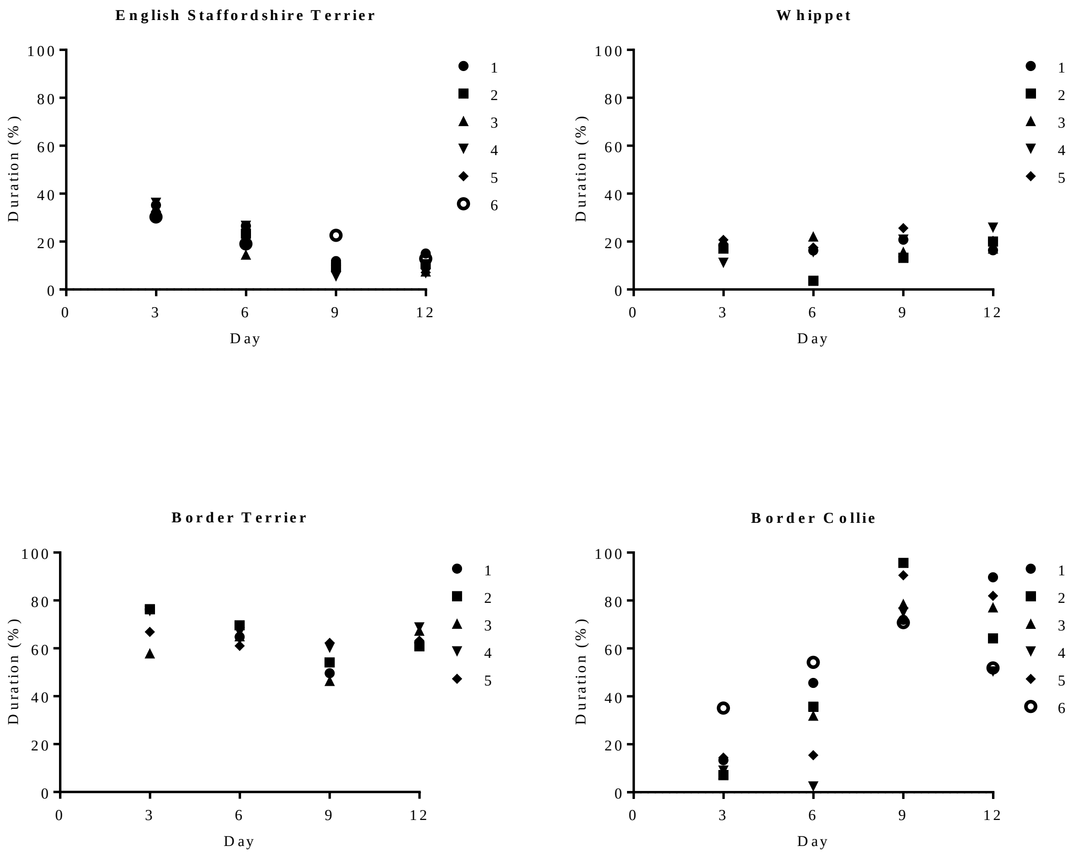


Figure 6c: Contact duration (%) per puppy on postnatal days 3, 6, 9 and 12. Results are separated per dam. Each puppy is represented by a different symbol.

4.3.3 Anogenital licking

There was a significant difference in the amount of time dams spent anogenital licking (p<0.001). The Labrador 1 dam spent significantly more time licking per puppy compared with other litters, except the Labrador 2 dam (Figure 7a, 7b and 7c). The Labrador 4 dam spent the least amount of time licking each puppy, which was significantly different to all other litters except Labrador 3. There was also a difference across observation days (p<0.001). On day 9, there was a significantly higher duration of anogenital licking compared with days 3, 6 and 12. There were no significant differences for anogenital licking within a litter (p=0.083).

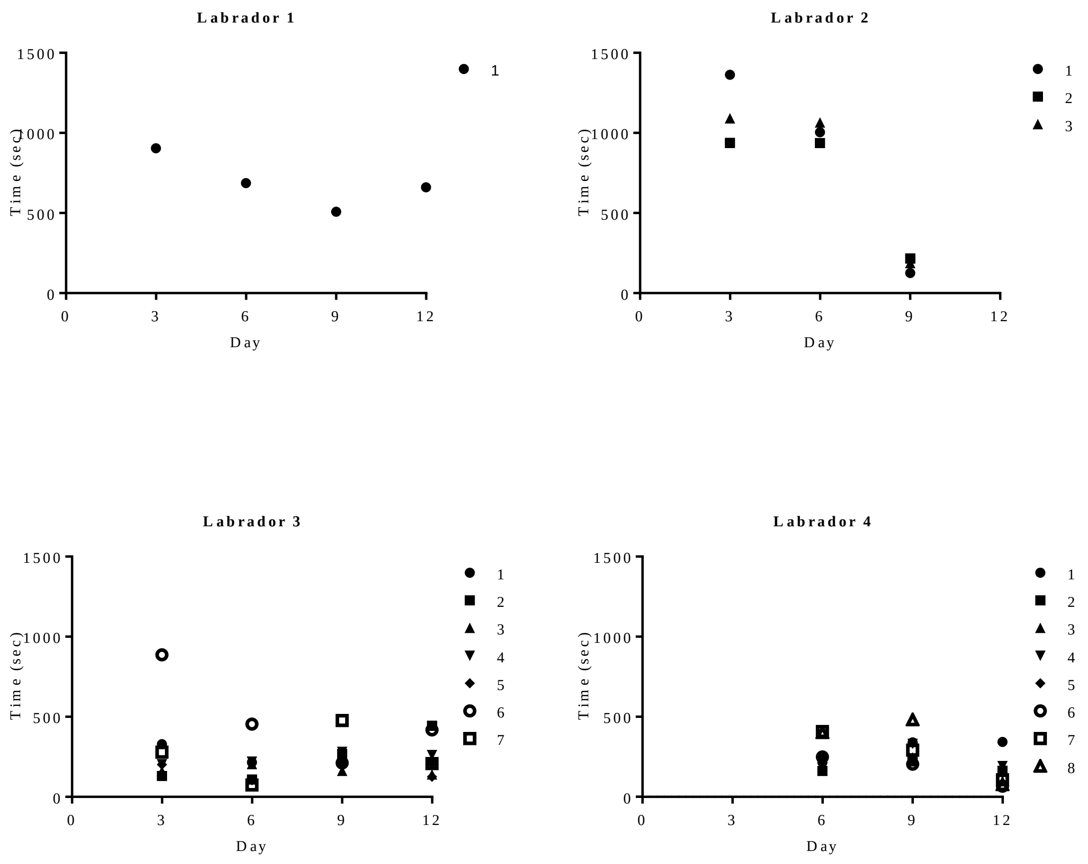


Figure 7a: Total anogenital licking amount (in seconds) per puppy on postnatal days 3, 6, 9 and 12 for Labradors. Results are separated per dam. Each puppy is represented by a different symbol.

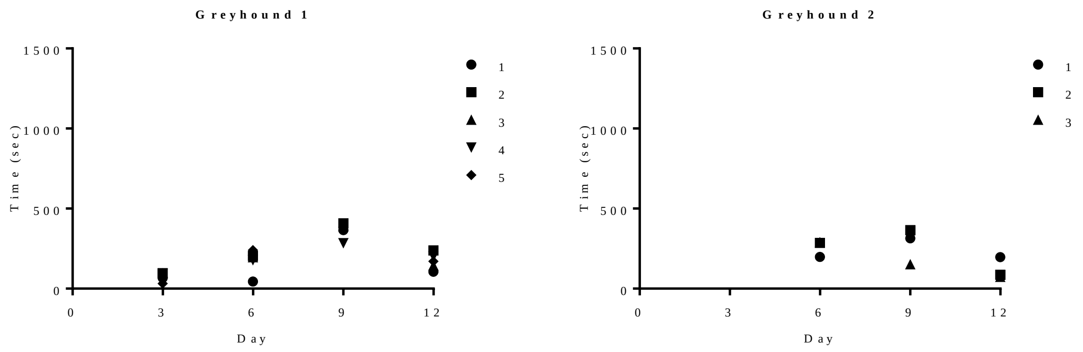


Figure 7b: Total anogenital licking amount (in seconds) per puppy on postnatal days 3, 6, 9 and 12 for Greyhounds. Results are separated per dam. Each puppy is represented by a different symbol.

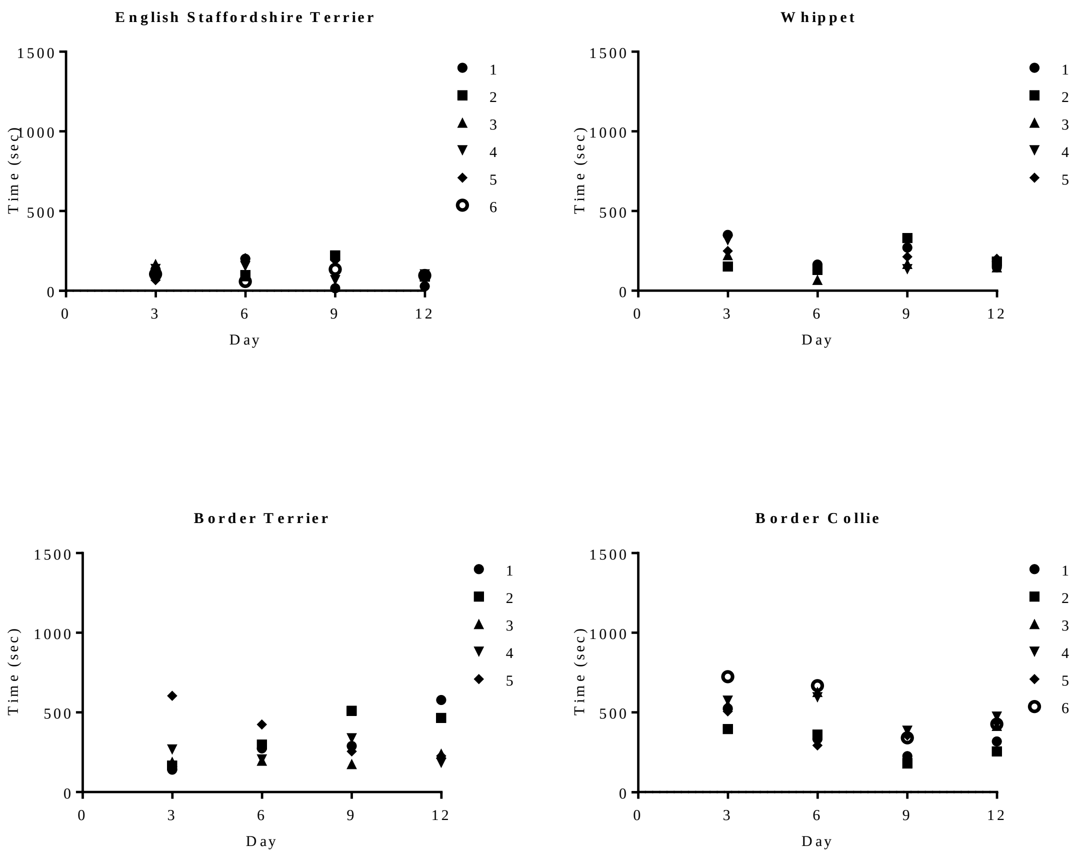


Figure 7c: Total anogenital licking amount (in seconds) per puppy on postnatal days 3, 6, 9 and 12. Results are separated per dam. Each puppy is represented by a different symbol.

Differences in anogenital licking duration and frequency were observed across days and between litters (Table 12). The smallest range in anogenital licking was observed in the Whippet litter on postnatal day 12, assuming a more evenly distributed licking amount for all puppies on this day (from 146.60 to 200.05 seconds). The largest range was noted in the Labrador 3 litter on postnatal day 3, of 130.84 to 887.48 seconds (difference of over 750 seconds, or 12.5 minutes).

Table 12: Anogenital licking frequency and duration across postnatal days 3, 6, 9 and 12 for 10 litters

Dam ID	Breed	Day	Puppy duration range (s)	Puppy frequency (n)	Total per litter (m)	(n)
1	English Staffordshire Terrier[a]	3	67.2-166.5	6-10	10.16	52
		6	57.7-203.8	4-10	13.43	41
		9	15.1-222.2	1-9	10.93	32
		12	27.6-103.1	0-2	8.42	3
2	Whippet[b]	3	151.4-350.1	11-27	21.51	84
		6	67.7-164.9	8-11	10.44	50
		9	136.0-331.00	11-28	18.67	84
		12	146.6-200.1	0-7	13.84	13
3	Greyhound 1[ab]	3	32.4-97.0	3-16	6.42	35
		6	44.8-241.1	7-14	14.39	49
		9	284.4-407.8	15-25	30.52	93
		12	105.9-238.5	7-11	14.33	46
4	Greyhound 1[cd]	6	197.8-289.3	17-23	12.87	58
		9	151.6-366.2	14-20	13.86	54
		12	72.8-197.00	9-14	5.93	34
5	Labrador 1[e]	3	903.8	41	15.06	41
		6	687.00	35	11.45	35
		9	507.8	28	8.46	28
		12	660.4	31	11.01	31

Table 12 continued

Dam ID	Breed	Day	Puppy duration range (s)	Puppy frequency (n)	Total per litter (m)	(n)
6	Border Terrier[c]	3	140.3-604.9	12-36	22.82	97
		6	197.2-423.8	13-30	23.32	93
		9	175.8-510.3	13-25	26.16	106
		12	184.6-577.8	12-29	28.27	90
7	Labrador 2[de]	3	938.6-1362.7	43-57	56.53	155
		6	936.5-1063.9	24-35	50.07	91
		9	125.4-216.6	8	8.82	24
8	Border Collie[ab]	3	395.2-724.6	31-49	54.57	227
		6	293.4-668.5	22-55	47.97	198
		9	178.9-385.2	9-28	27.79	117
		12	256.0-474.0	15-38	38.85	167
9	Labrador 3[f]	3	130.8-887.5	13-32	36.53	139
		6	74.1-453.8	8-21	23.00	98
		9	161.9-477.2	14-30	30.38	150
		12	125.6-444.7	9-24	30.24	125
10	Labrador 4[f]	6	162.5-407.9	7-19	34.76	90
		9	204.6-481.9	10-23	40.90	115
		12	66.9-343.5	7-17	20.89	82

Differences in letters next to dam breed indicate a significant difference in anogenital licking between dams across the total observation period.

4.4 Discussion

This is the first study to provide detailed analysis of maternal behaviours within and between domestic dog litters in the first two postnatal weeks. It is within this period that puppies require intensive maternal care as they cannot regulate their own body temperature or urinate and defecate independently (Kendrick et al., 1997). However, they also develop rapidly and the level of maternal care decreases accordingly (Rheingold, 1963; Malm and Jensen, 1997). This did occur within the current study as nursing and anogenital licking were significantly different across observation days. The first aim was to determine whether there were differences within a litter for maternal care behaviour. No significant difference was found within a litter for observed behaviours, which may be attributed to sample size of the current study. Nonetheless, these results suggest that dam maternal behaviour is distributed equally across all puppies and therefore the dam does not favour or exclude any individuals. This is a positive observation for dog breeders as they can choose dams exclusively on the maternal care she gives to the litter rather than having to observe dam interaction with each individual puppy.

4.4.1 Nursing

Differences in the amount of time each dam nursed across days were apparent. Previous research has documented nursing behaviour in the first two postnatal weeks in domestic dogs (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016). In the current study, the duration of nursing for each puppy ranged from 0, meaning the puppy did not nurse during the observation period, to 65%. This may be due to the short sampling period which may have missed the dam being in the whelping box nursing her puppies. Previous studies have found a larger range in nursing. Studies observing litters using a short sampling method (Rheingold, 1963: one hour; Scott and Fuller, 1965: 10 minutes; Guardini et al., 2015: 15 minutes) have documented nursing to occur between 0 and 100% (7-87%, 13-83% and 0-100%, respectively). Studies determining nursing using a longer time period (i.e. >12 hours) have detailed a shorter range in nursing (Korda and Brewinska, 1977b: 50-86%; Foyer et al., 2016: 8-23%). In the current study, nursing duration was similar to results presented by Grant (1987) who used a one hour period to observe dam behaviour where nursing ranged from 20-63%. Differences in results may be in part due to different breeds and number of dogs observed. In previous studies, litter sizes observed ranged from one (Grant, 1987) to 24 (Scott and Fuller, 1965). Breed type also differed between studies from small (Fox Terrier in Scott and Fuller, 1965) to large dog breeds (i.e. Boxers used by Guardini et al., 2015), and within studies where both short-haired Dachshund and German Shepherds were observed (Guardini et al., 2015). The effect of breed on nursing behaviour is as yet unknown. Foyer et al. (2016) was the only study to observe litters of a single breed the German Shepherd. It is also possible that suckling was not only undertaken by puppies for nutrients, but also for comfort. Puppies requiring comfort may nurse for comfort rather than for nutrients, although this may not have been reflected in the results as it would have been difficult why the puppy was nursing.

Differences between current and previous data may also be due to coding methodology. A one hour period across a 24-hour period was used to observe puppies in this study. In Chapter 3, a one hour period was found to be representative of a 24-hour period, thus there should not be differences between studies using a one hour period and studies using a longer period of observation. This was not always the case and differences may be due to variances in the ethogram. In the current study, each puppy was identified and recorded when they were suckling, as opposed to when the dam was nursing. All studies, excluding Foyer et al. (2016), described the amount of nursing per dam. Foyer et al. (2016) observed dam nursing behaviour and averaged the amount of nursing for the number of puppies in the litter. It is expected that a smaller percentage of nursing was observed as each puppy suckling at different times is shorter in duration than the time the dam feeds. To be more confident with these results found in the current study, a repeat study using the same ethogram as the current study and a larger sample size is necessary. Understanding differences in dam nursing behaviour allow the breeder to intervene with the dam and her puppies. Dams likely to nurse for a shorter period of time may need the breeder to keep the animal in the whelping box while she is feeding and this may differ between breeds and bitches.

Duration of nursing was higher on postnatal days 3 and 9 in this study. The duration of nursing decreased across days within the first twelve postnatal days in some previous studies (Scott and Fuller, 1965; Korda and Brewinska, 1977b; Foyer et al., 2016). Rheingold (1963) reported an increase in nursing until day 5, while Grant (1987) observed an increase in nursing until day 12. There was also an increase in nursing observed between primiparous and multiparous bitches (Guardini et al., 2015). Maternal care is not likely to dramatically decrease between the first two postnatal weeks as puppies are still immature and require nursing for survival (Freedman et al., 1961; Scott and Fuller, 1965).

4.4.2 Puppy lying with another individual

The duration of time a puppy spent in contact with the dam or another puppy was significantly different between dams, but not different across observation days. Contact within domestic dog litters has been documented during the first two postnatal weeks (Rheingold, 1963; Korda and Brewinska, 1977a; Guardini et al., 2015; Foyer et al., 2016). Contact behaviour in the current study was observed between 0-100% of the time during the first 12 postnatal days. As the current study recorded puppy contact occurring between dam and other puppies, and other studies only documented contact between mother and puppy, differences in study results were expected. However, this did not occur. When a longer period of time was used to observe contact behaviour, a shorter range of contact was observed. Korda and Brewinska (1977a) observed puppies for 14 continuous hours and documented between 29-86% contact, while Foyer et al. (2016) described 10-32%. For the latter study contact was observed when the dam was lying with any puppy and then averaged across litter number. Accuracy of the data may be impaired where there are differences in contact duration per puppy.

Within the current study there were no significant differences across observation days for contact duration. Rheingold (1963), Korda and Brewinska (1977a) and Foyer et al. (2016) documented a decrease in mean duration of contact across time, while Guardini et al. (2015) observed an increase. This may be due to differences in the ethogram used to code contact behaviour. The definition of lying/contact may influence how these behaviours were coded and influence comparisons. Rheingold (1963) and Guardini et al. (2015) described contact behaviour as physical contact between puppy and dam in the whelping box, while Korda and Brewinska (1977a) coded contact when the dam was in direct tactile contact with a puppy. Finally, Foyer et al. (2016) described contact behaviour as the duration in which the dam was lying in the whelping box with her elbows on the floor and in physical contact, excluding tail and limbs, with at least one puppy. The current study described contact as 'when a puppy is in a lying position and has at least 50% of its body against or alongside another puppy or the dam.' When a puppy is moved by the dam's nose or licked on the head, other studies have classified this as contact, however, the description was purposefully changed in the current study to reflect the amount of time a puppy is seeking/acquiring necessary contact (i.e. thermoregulation). Warmth for a puppy is only achieved if a significant amount of the puppy's body is in contact with another individual. Therefore, the results presented in the current dataset are likely to accurately reflect occurrences of a puppy seeking others for development and warmth. In the case of a small litter and puppies not receiving warmth, it may be necessary for the breeder to supply puppies with a covered warm water bottle or something similar to imitate warmth received from another individual.

Contact is essential for young puppies (Kendrick et al., 1997). It is therefore important to determine ambient temperature when observing contact behaviour. In high ambient temperatures it is most probable that puppies may disperse and lay alone. Temperature data have yet to be reported in other studies, but ambient temperature was collected in the current study. The highest ambient temperatures on observations days 3, 6, 9 and 12 were documented in the Labrador 2 (day 3) and Labrador 4 (days 6, 9 and 12) litters (Table 8). Only on day 3 were puppies in the Labrador 2 litter observed, on average, to lay in contact for a longer duration compared to other litters. The lowest ambient temperatures on observation days 3, 6, 9 and 12 were seen in the Border Collie (day 3, 6 and 12), and Greyhound 1 (day 9) litters (Table 8). Only on day 12 the litter with the coldest ambient temperature showed increased contact behaviour duration. It is most likely that puppies in a higher temperature environment are able to source environmental heat, rather than heat from other puppies or the dam, while puppies in an environment with a cooler ambient temperature seek others for contact to remain at a stable internal temperature. However, this was not observed within the current study. Contact duration may also reflect litter size, where puppies in a larger litter are more likely to be in contact with a puppy or the dam, as compared to a smaller litter. Unfortunately, due to the small sample size of the current dataset it was not possible to determine whether litter size or ambient temperature affected contact duration.

4.4.3 Anogenital licking

The amount of anogenital licking a puppy receives can influence brain development, in particular HPA axis activity (Greenough, 1990; Lenz and Sengelaub, 2009). Significant difference in anogenital licking duration between dams was observed in the current study.

There was also a difference in the total amount of licking observed between similar litter sizes, providing evidence that litter size is not predictive of total licking amount per puppy. With a larger dataset, additional factors (i.e. litter size, puppy sex) may be used in a model to determine whether these factors have an effect on anogenital licking duration.

Anogenital licking is a low duration and infrequent behaviour. Each litter was observed for 12 continuous hours during the day. In this dataset the longest duration of anogenital licking observed was 22.7 minutes for a single puppy and 56.5 minutes for a litter occurring on one observation day. The longest duration of anogenital licking across the combined observation days for a litter was almost three hours (169.2 minutes), which was observed in the Border Collie litter. Three studies identified anogenital licking duration (Rheingold, 1963; Guardini et al., 2015; Foyer et al., 2016) and two others described anogenital licking frequency (Korda and Brewinska, 1977a; Grant, 1987). As Rheingold (1963) and Guardini et al. (2015) observed litters for a short time period, it may be possible that the results do not reflect the behaviour over a 24-hour period. Across the four observation days (postnatal days 3, 6, 9 and 12) the longest total duration of anogenital licking was found to be 5.6% on postnatal day 6 for the black Sheltie (Rheingold, 1963). This result was lower than that found in the current study (7.32% in the Greyhound litter on postnatal day 9). Guardini et al. (2015) reported a larger maximum duration of anogenital licking with approximately 33% for total litter licking observed on postnatal day 10. Foyer et al. (2016) observed litters for 12 hours (every second hour during a 24-hour period) and the maximum duration of anogenital licking observed was 5% for a puppy on one observation day, similar to the current study. When observing the frequency of licking it was found that a 12-hour period at night or during the day would represent licking frequency of a 24-hour period (refer to Chapter 3). The highest number of licking episodes observed was 106 within a litter of five puppies. Korda and Brewinska (1977a) reported behaviour for 14 continuous hours and observed a maximum number of anogenital licking acts of 110 for a litter of four; similar to the amount of licking observed in the current study. Grant (1987) documented anogenital licking across a one hour period and observed a maximum of 37 licking acts. A short sampling period to record anogenital licking frequency was not representative of a 24-hour period, therefore the results presented by Grant (1987) may not be accurate. Using observations in the current study, licking acts were observed between 0 and 57 across a 12-hour period. Thirty-seven licking acts in an hour period, reported by Grant (1987) seems relatively high.

Anogenital licking was significantly different across observation days in this study, results supported by Rheingold (1963) and Guardini et al. (2015). Maternal care behaviour in regards to anogential licking is expected to change following the first two postnatal weeks as puppies have not been observed to urinate or defecate independently before postnatal day 14 (Lawler and Chandler, 1992). Rheingold (1963) observed two peaks in mean data for anogenital licking: a peak on day 6 and again on day 12. Although the peaks differed between the current study and Rheingold's (1963) study, fluctuations in both datasets occurred. However, Korda and Brewinska (1977a) and Foyer et al. (2016) observed a decrease in anogenital licking across four postnatal days (3, 6, 9 and 12). A varying pattern, but predominant decrease, in anogenital licking was also observed by Grant (1987), while Guardini et al. (2015) documented an increase in mean licking duration over postnatal days 1 to 21. It is likely that reduced amount of anogenital licking over the first few postnatal weeks is common as puppies are becoming able to urinate and defecate for themselves. The pattern of licking across the first two postnatal weeks may not be as important as total anogenital licking duration.

There was no significant difference observed in the duration of anogenital licking within a litter in this study, although data were approaching significance (*p*=0.08). A small sample size was used in this study and a larger dataset may determine whether inter-variation differences occur. The total difference between the least and most licked puppies differed between litters (Table 12). There was less than a 60 second difference between lowest and highest licked puppy in the Whippet litter on postnatal day 12, while there was over a 12 minute difference between the lowest and highest licked puppy in the Labrador 3 litter on postnatal day 3. The duration of anogenital licking was not always stable within a litter across observation days in the current study. On some days, one puppy received more licking than others, suggesting that the dam does not lick her puppies for an equivalent duration. Although differences between dams in anogenital licking was observed, the minimum amount of licking needed to stimulate defecation and urination is unknown in domestic dogs.

It is often difficult to determine puppy feeding within the first few postnatal days as the puppies are small and at times puppies fall asleep on the nipple but it may look like they are feeding. Daily observations within the first 12 postnatal days may be necessary to determine changes and differences in nursing behaviour across days. The ability to determine individual puppies in the current study was more difficult on postnatal day 3 compared to other observation days as puppies are small during the first few postnatal days. At times, puppies could not be observed as the dam curved her body around them or they were underneath another puppy. Future studies should include better techniques to identify puppies (chalk marking).

4.5 Conclusion

This Chapter detailed the first comprehensive observations of differences in maternal care behaviour in 10 domestic dog litters in the first 12 postnatal days. It provides evidence that not all dams provide the same level of maternal care. Anogenital licking can alter HPA axis development and therefore the dam may be altering the puppy's ability to appropriately respond to stressors later in life. If dog breeders consider dam mothering ability prior to re-breeding, selected dams may be more likely to produce puppies which respond less stressfully in certain situations (situations not needing a fight or flight response), as discussed in Chapter 2. Puppies with a lower stress-threshold are more likely to become problematic to new owners and therefore may be relinquished to a shelter. As there were differences in maternal care behaviour between litters, but not within litters, it is necessary to determine whether these differences affect puppy stress response later in life. Hence, in the next Chapter the impact of maternal care on puppy stress response behaviour is discussed.

Chapter 5. Stress-related responses in domestic dog puppies

The amount of maternal care, in particular licking, alters the stress response of offspring in laboratory rats. Differences in maternal care behaviour in 10 domestic dog litters were described in Chapter 4. In this Chapter, the link between maternal care and future offspring behaviour is explored. Specifically, puppies were tested at seven weeks of age to determine whether maternal care received by puppies affects behaviour and physiology.

5.1 Introduction

There is a known relationship between maternal care and offspring behaviour. In rats, the amount of licking and grooming received can alter pup behaviour later in life. Specifically, offspring licked more when young show a number of behavioural differences including reduced fear response to novelty as adults (Liu et al., 1997, Caldji et al., 1998), an increase in exploration and searching in tests, a shorter swim path to a selected target, decreased startle response and shorter latency to eat when provided food (Caldji et al., 1998). Differences observed were due to changes in the HPA axis, peripheral nervous system and central nervous system (Caldji et al., 1998, Caldji et al., 2000). There is strong indication that maternal care in the first 10 postnatal days has a large impact on the HPA axis in rats (Levine and Lewis, 1959; Meaney and Aitken, 1985; Myers et al., 1989). Therefore, this period was targeted for observation in domestic dogs. As both the rat and dog are altricial species and their developmental stages are similar (refer to Chapter 2 for further information), it is likely that the underlying physiological and behavioural repercussions of differential maternal care occur in the rat and domestic dog.

Maternal care and consequent puppy behaviour has been documented previously (Tiira and Lohi, 2015; Foyer et al., 2016; Guardini et al., 2016), as described in Chapter 2. However, inconsistencies with the methodology used within these studies make it difficult to determine the link between maternal care and subsequent behaviour. For example, in the study by Tiira and Lohi (2015) the owner may not have known the mothering style of the dam, or may have inaccurately interpreted the behaviour of the dam, or maternal care may not have been thoroughly observed in the first two week postnatal period (every second hour for an hour, once a week during the first three postnatal weeks – refer to Chapter 3). In the study undertaken by Foyer et al. (2016) tests were conducted at different sites and dogs were housed with foster carers until temperament tests which may have impacted behavioural responses. A short observation period (15 minutes) of puppy-dam dyad was used to document maternal care, and, for analysis, a combined maternal care score rather than individual behaviours were used to identify any association with later puppy stress-related behaviour. In addition, these studies documented maternal care at the litter level, rather than puppy level.

Novelty and isolation tests can induce stress-related behaviours in animals (Dickerson and Kimeny, 2004). Vocalisation and activity levels can be altered when a puppy is separated from its mother and littermates (Ross et al., 1960; Elliot and Scott, 1961; Wilsson and Sundgren, 1998a; Wilsson and Sundgren, 1998b; Gazzano et al., 2008). Vocalisation and exploratory behaviour during isolation differs between dogs reared under opposing conditions (Elliot and Scott, 1961; Davis et al., 1977; Wilsson and Sundgren, 1998a; Wilsson and Sundgren, 1998b; Gazzano et al., 2008). In the arena test, which evaluates reactions of puppies in a novel environment where an unknown passive human is present, puppies raised by more experienced mothers (multiparous dams) produce puppies with a shorter latency time to shriek, were more competitive and active, and spent more time within the circle (a 2m circle painted in the middle of the 3.6m^2 arena)

(Wilsson and Sundgren, 1998b). This suggests that puppies mothered by more experienced mothers were less afraid to be near the novel human. However, the association between maternal care and stress behaviour in isolation have produced contrasting results (Wilsson and Sundgren, 1998b; Foyer et al., 2016; Guardini et al., 2016). Behaviour has been compared in puppies housed at a breeder facility or with a foster family, with varying levels of human handling (Gazzano et al., 2008). Puppies raised in a family environment, both handled and non-handled, had a shorter latency to vocalise ($p<0.05$) compared to the puppies raised in a breeding kennel. Vocalisation duration also differed; puppies raised in a professional breeding kennel had a shorter vocalisation duration compared to puppies raised with a family ($p<0.05$). Locomotor activity during isolation was significantly greater in handled puppies compared to non-handled puppies in the same location (Gazzano et al., 2008).

Maternal care as a predisposing factor for dog anxiety is a relatively new concept. The aim of this study was to test the assumption that a link exists between maternal care and puppy stress response when puppies were seven weeks old. The hypothesis is that puppies receiving higher amounts of anogenital licking on postnatal day 6 would display a reduced stress response (i.e. reduction in vocalisation and heart rate, and an increase in activity). The experiment was conducted by comparing previously coded maternal behaviour (described in Chapter 4) and behaviour of seven-week old puppies during an isolation test.

5.2 Methods

5.2.1 Animal subjects

A total of 49 puppies from 10 litters participated in the study (see Table 13). Puppies were tested at approximately seven weeks old, between December 2014 and July 2015. Isolation tests were performed at the breeder's residence, with all breeders (n=10) located in South Australia, Australia. Approval from the University of Adelaide Animal Ethics Committee was obtained (S-2014-098).

Table 13: Details of puppies involved in isolation testing

Litter ID	Breed	Dam Parity	Puppy Id	Birth Order	Sex*	Birth weight	Weight at 7 weeks
1	English Staffordshire Terrier	1	1	1	M	187	3200
			2	2	M	239	3800
			3	3	F	234	3700
			4	4	M	188	4700
			5	5	F	245	4400
			6	6	F	184	2900
2	Whippet	3	1	1	M	309	3600
			2	2	F	279	2400
			3	3	F	310	3600
			4	4	M	306	3600
			5	5	M	257	3600
3	Greyhound	4	1		M	n/a	6400
			2	n/a	M	n/a	6300
			3	Caesarean	M	n/a	5900
			4	birth	M	n/a	6500
			5		M	n/a	6000
4	Greyhound	1	1	n/a	F	700	7100
			2	Caesarean	M	720	7900
			3	birth	F	450	6900
5	Labrador	3	1	1	F	700	8100
6	Border Terrier	2	1		F	236	1800
			2	n/a	M	258	2200
			3	Caesarean	F	218	1700
			4	birth	M	262	1800
			5		F	234	1800

*Sex: M=male and F=female.
n/a: not available; a caesarean birth did not allow birth order to be recorded.

Table 13 continued

Litter ID	Breed	Dam Parity	Puppy ID	Birth order	Sex*	Birth weight	Weight at 7 weeks
7	Labrador	1	1	1	F	640	7200
			2	2	M	618	7800
			3	3	F	513	6500
8	Border Collie	1	1		M	270	3000
			2		M	340	3500
			3	n/a	M	360	3700
			4	Caesarean	M	340	3500
			5	birth	F	420	4100
			6		F	310	2900
9	Labrador	1	1	1	M	460	5500
			2	2	F	450	5000
			3	3	F	450	5100
			4	4	F	440	4200
			5	5	M	460	4800
			6	6	M	470	4700
			7	7	F	500	5600
10	Labrador	3	1	1	M	562	6700
			2	2	M	518	6900
			3	3	M	505	6900
			4	4	F	457	6400
			5	5	M	528	6600
			6	6	F	456	6600
			7	7	F	434	6000
			8	8	M	541	6700

*Sex: M=male and F=female.
n/a: not available; a caesarean birth did not allow birth order to be recorded.

5.2.2 Procedure

Isolation tests were conducted at the breeder's residence between 0900 and 1300 hours. The isolation box was positioned outdoors, but under a roof (either a pergola, veranda or carport) such that the puppies were not able to view movement in the sky (e.g. birds, tree branches, planes) and to reduce weather influence. A 48 inch square (120cm L x 120cm W x 120cm H) indoor/outdoor exercise pen (T.F.H Australia) was used as the isolation box (see Figure 8). Dark vinyl material covered the entire length and height of the isolation box and was secured to the isolation box at intervals using cable ties. The isolation box was placed onto a vinyl sheet marked with silver duct tape, separating the floor into $40cm^2$ squares, resulting in nine even-sized squares (Figure 9). A ball was placed into the 6^{th} square and a plastic water bowl, half filled with water, was placed into square 1. The isolation door opening was positioned at the corner of square 9. A camera (Sony, HDR-PJ10E) was secured to a tripod, placed on a box and focused to view the entire isolation box floor, similar to that in Figure 9.

Figure 8: Set up of the isolation box ready for the puppy to enter and begin testing.

Figure 9: The vinyl floor of the isolation box marked into 9 squares. The plastic water bowl was placed on the first square while the plastic ball was placed within the 6th square. Every puppy was placed in the middle square (5th square) to begin testing.

5.2.3 General procedure

The order in which puppies undertook the isolation test was randomised using a random number application (Random Number Generator, Skytrait) (Table 14). Each puppy was individually removed from the dam and litter to be weighed (Bathroom Scale, Target). Some litters were kept away from the dam while all puppies were tested, but for other litters the dam remained with puppies not undergoing testing. Each puppy was held while its heart rate was measured (Figure 10) by stethoscope (Littmann, 3M) and was with the researchers for approximately three minutes before being placed in the middle square of the isolation box. The opening was secured shut with two bolt snaps, one at the top of the enclosure and one towards the bottom of the enclosure. The excess vinyl was then pulled across the opening, to ensure the puppy could not see out of the box, and secured with duct tape. As soon as the vinyl was secured with duct tape, the timer was started, and the puppy remained in the box for four minutes. The researchers remained still and quiet during the procedure and out of sight of the puppy.

When the puppy had been in the isolation box for three minutes, a 1200mm stainless steel round pet food bowl (Show Master, Aus) was dropped, approximately 500mm from the ground next to the opening area of the box. To ensure the noise made was consistent across all testing locations, the bowl was dropped onto a porcelain tile (3000mm^2). At the four minute mark, the duct tape was removed from the vinyl and the bolt snaps were removed from the enclosure to allow the door to open. The researcher opened the enclosure door and the puppy was picked up and had its heart rate taken using the previously indicated technique. The puppy was then returned to the litter. The total time each puppy was removed from the litter was approximately eight minutes. Urine and faeces produced by puppies during the isolation test were removed with water and paper towel before testing the next puppy.

Table 14: Puppy testing order for the isolation test

Litter Id	Puppy Id	Order of testing
1	1	6th
	2	3rd
	3	4th
	4	2nd
	5	5th
	6	1st
2	7	4th
	8	3rd
	9	1st
	10	5th
	11	2nd
3	12	2nd
	13	5th
	14	3rd
	15	1st
	16	4th
4	17	1st
	18	3rd
	19	2nd
5	20	-
6	21	1st
	22	4th
	23	2nd
	24	5th
	25	3rd
7	26	2nd
	27	1st
	28	3rd
8	29	6th
	30	3rd
	31	5th
	32	1st
	33	2nd
	34	4th
9	35	3rd
	36	5th
	37	7th
	38	4th
	39	2nd
	40	1st
	41	6th
10	42	4th
	43	5th
	44	1st
	45	6th
	46	3rd
	47	7th
	48	8th
	49	2nd

Figure 10: Positioning of the stethoscope on the puppy to determine heart rate.

5.2.4 Behavioural responses to isolation

Video footage from the isolation test was viewed, and puppy behaviour coded to determine vocalisation and exploration behaviours. The duration and frequency of behaviours were coded using Mangold INTERACT software (v.9). Behaviours observed are detailed in Table 15. Vocalisations (Fuller, 1955; Scott and Fuller, 1965), explorative activity (LeDoux, 1986) and heart rate (Murphree et al., 1967; Fox, 1978; Beerda et al., 1998; Palestrini et al., 2005) are indices of an animal's emotional state and were therefore included as essential behaviours to record. As the observation period was equal for all puppies (three minutes), behaviour duration was expressed as seconds. Behaviours coded as frequencies were expressed as such. To assess stress-related responses, behaviours were compared between litters. Two puppies (6 and 43; Table 14) were removed from behavioural and heart rate analysis as there were technical problems with the recorded data during the time the puppies were in isolation.

Table 15: List of behaviours assessed during the isolation test

Behaviour	Description
Latency to first vocalisation	The first time the puppy emitted a noise from their mouth after the stop watch was started. Noises included whines, barks, howls, yelp or cries
Duration of vocalisation	The total time the puppy made a noise from their mouth
Duration of activity	The total time the puppy was moving, where it had at least one paw off of the ground
Duration in opening square	The total time the puppy was within the opening square (9th square) of the isolation box. The puppy had to have at least 50% of its body within the square before it was recorded as within the opening square
Number of lines crossed	Each time a puppy crossed a line (duct-tape on the floor) with their two front paws
Duration of drinking	The time the puppy spent drinking water from the bowl
Duration of interaction with the ball	The time the puppy was interacting with the ball. This included pawing at, moving the ball with their nose or holding/chewing the ball in their mouth
Latency to move after the bowl is dropped	The duration taken by the puppy to move at least one paw off of the ground after the bowl is dropped
Latency to vocalise after the bowl is dropped	The time taken for the puppy to emit a noise from their mouth after the bowl is dropped on the floor. Noises included whines, barks, howls, yelp or cries

5.2.5 Statistical analysis

5.2.5.1 Linear mixed model

Data were analysed using SPSS (IBM, v.23). Non-normally distributed data for puppy behaviour in the isolation box were identified and log transformed. Transformed behaviours included opening square duration, duration drinking, duration with the ball, vocalisation latency, duration of vocalisation and heart rate. A linear mixed model was developed to determine the association of factors regarding puppy behaviour observed in the isolation box test. The model included fixed effects of dam parity (primiparous or first time dams, multiparous or dams which have had a previous litter), breed size (small, large), birth type (natural, caesarean) and puppy sex (female=bitch, male=dog). The dam was included as a random term, while anogenital licking (postnatal day 6) and puppy birth weight were adopted as covariates. Breed size was divided into two groups, small and large, as portioning the groups into three groups would have reduced the numbers of puppies within each group further.

Anogenital licking on day 6 was chosen to represent maternal behaviour as 1) all litters were observed during this period, and 2) there was no significant difference (p=0.943) between anogenital licking on postnatal days 6 and 9, using bivariate correlation in SPSS (IBM, v.23).

The residual vs predicted value plots were observed for each behaviour to determine that no pattern was occurring in the data. A model for each behaviour in isolation was conducted and type 1 sum of squares was used to account for certain factors first. Main effects were listed in order of birth weight, breed size, parity, birth type, sex and anogenital licking day 6. Two-way interactions were then added as follows: breed size by anogenital licking day 6, parity by anogenital licking day 6, birth type by anogenital licking day 6, and sex by anogenital licking day 6. The model for each behaviour was first analysed using factors in the order presented above. Non-significant ($p>0.05$) factors were then removed one at a time until a factor showed significance, or until all factors were removed. The more complex factors (two-way interactions) were removed first, followed by less complex factors (main effects). Significance was accepted at $p<0.05$. Due to the small dataset, factors close to significance acceptance point remained in the results to allow for discussion within results. Data are presented as mean ± standard deviation of the mean, unless indicated otherwise.

5.3 Results

5.3.1 Duration of activity

Duration of puppy activity in the isolation test varied from 53.0 to 145.8 seconds (103.0 ± 3.1 seconds). No factors were significantly associated with puppy activity duration within the test. However, breed size was approaching significance ($p=0.063$; see Table 16).

Table 16: The effect of dam and puppy variables on the behaviour and heart rate in the isolation test

Factors	Activity duration	Duration in opening square	Lines crossed	Duration drinking	Ball duration	Latency to vocalise
Birth weight	ns	0.840	0.172	ns	0.683	ns
Breed size	0.063	0.223	0.113	ns	0.659	ns
Parity	ns	0.481	0.673	ns	0.612	ns
Birth type	ns	0.109	0.543	ns	0.525	**0.054**
Sex	ns	0.721	0.185	ns	0.218	ns
Anogenital lick day 6 (ALd6)	ns	0.430	0.366	ns	0.183	**0.009**
Breed size * ALd6	ns	**0.046**	**0.025**	ns	ns	ns
Parity * ALd6	ns	ns	ns	ns	ns	ns
Birth type * ALd6	ns	ns	ns	ns	**0.011**	ns
Sex * ALd6	ns	ns	ns	ns	ns	ns

Table 16 continued

Factors	Vocalisation duration	HR pre-test	HR post-test	HR difference	Latency to move post-noise	Latency to vocalise post-noise
Birth weight	ns	ns	ns	0.685	0.385	ns
Breed size	ns	ns	ns	0.806	0.134	ns
Parity	ns	ns	ns	0.555	0.389	ns
Birth type	ns	ns	ns	0.776	**0.005**	ns
Sex	ns	ns	ns	0.700	0.831	ns
Anogenital lick day 6 (ALd6)	ns	**0.044**	ns	0.370	0.073	ns
Breed size * ALd6	ns	ns	ns	ns	0.175	ns
Parity * ALd6	ns	ns	ns	**0.039**	ns	ns
Birth type * ALd6	ns	ns	ns	ns	**0.035**	ns
Sex * ALd6	ns	ns	ns	ns	ns	ns

5.3.2 Duration in opening square

Puppies spent between 4.2 and 145.9 seconds in the opening square (51.1 ± 5.3 seconds). There was significant association between the two-way interaction of breed size and anogenital licking on day 6 and puppy duration in the opening square (p=0.046). A one second increase in anogenital licking in large breeds resulted in a 0.32 second increase in duration spent in the opening square of the isolation box (Figure 11). The mean for both breed sizes were similar; duration within the opening square for small breed puppies was 42.6s ± 22.4s (n=15) while for large breed puppies the mean was 43.2s ± 24.2s (n=32) (Table 17).

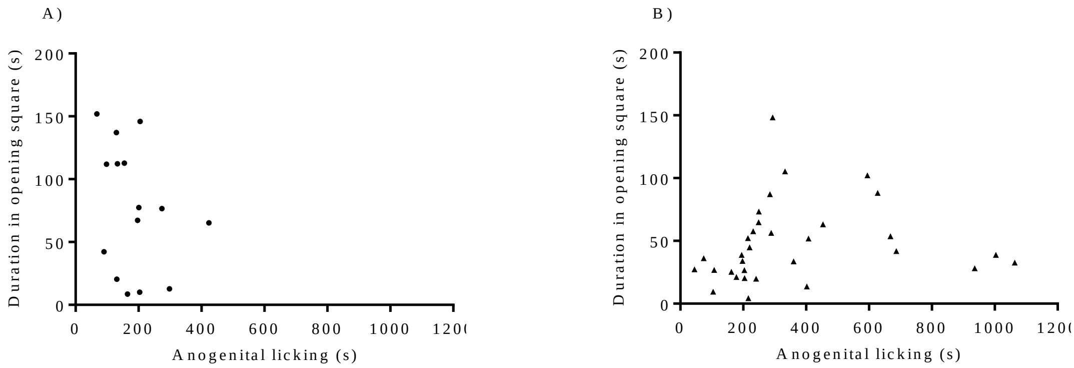


Figure 11: Duration in opening square as a function of amount of anogenital licking on postnatal day 6 for A) small and B) large dog breeds. Each symbol represents an individual puppy in each group.

Table 17: Association of breed size, parity and birth type on the behaviour of puppies in the isolation box

Variable (n)	**Activity duration (sec)**	**Duration in opening square (sec)**	**Lines crossed**	**Duration drinking (sec)**	**Ball duration (sec)**	**Latency to vocalise (sec)**	**Vocalisation duration (sec)**
Breed size							
Small (15)	110.8±15.1	42.6±22.4	55.3±11.9	2.2±3.3	2.7±1.6	31.3±25.1	75.6±44.6
Large (32)	106.0±21.0	43.2±24.2	61.6±19.1	7.7±11.1	14.5±21.5	30.6±37.7	107.5±51.0
Parity							
1st parity (28)	104.9±21.4	44.6±23.8	59.9±19.6	8.3±10.8	14.4±21.6	31.7±36.8	110.8±49.4
2 or more parities (19)	113.5±11.3	39.4±23.2	59.3±11.2	0.8±0.8	3.1±2.2	28.7±28.7	68.1±40.6
Birth type							
Natural (28)	102.9±19.5	54.0±42.9	52.6±23.6	2.5±6.7	3.2±8.9	32.4±30.7	97.3±39.6
Caesarean (19)	104.1±26.4	60.1±37.1	45.6±13.4	0.5± 0.9	20.1±42.8	13.8±18.0	122.5±47.4

Table 17 continued

Variable (n)	**HR pre-test (bpm)**	**HR post-test (bpm)**	**HR difference (bpm)**	**Latency to move post noise**	**Latency to vocalise post noise**
Breed size					
Small (15)	168.0±14.4	194.7±18.0	26.7±4.6	3.4±0.89	3.5±0.50
Large (32)	166.3±18.3	192.6±9.9	26.3±17.7	3.3±0.72	5.3±0.93
Parity					
1st parity (28)	165.1±17.5	190.9±7.6	25.7±17.7	2.6±0.51	5.5±1.07
2 or more parities (19)	170.7±16.2	198.7±19.7	28.0±4.0	4.4±1.15	3.6±0.33
Birth type					
Natural (28)	167.5±20.7	198.5±16.4	31.0±17.5	2.2±0.56	5.4±1.07
Caesarean (19)	177.9±21.9	208.7±29.9	30.8±25.9	5.0±1.09	3.7±0.36

5.3.3 Number of lines crossed

Puppies crossed between eight and 96 lines during the isolation test (50 ± 3 lines). There was a significant association between the two-way interaction of breed size and anogenital licking on day 6 and number of lines crossed (*p*=0.025). Every ten second increase in anogenital licking of puppies for large breeds resulted in a decrease in lines crossed by 1.3 lines (Figure 12).

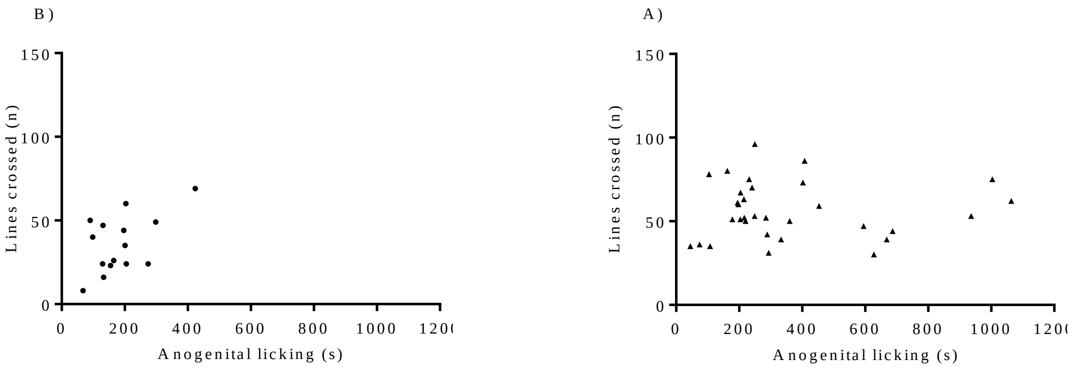


Figure 12: The number of lines crossed as a function of the amount of anogenital licking on postnatal day 6 for A) small and B) large dog breeds. Each symbol represents an individual puppy in each group.

5.3.4 Duration of drinking

Duration of drinking in the isolation test varied from 0.0 to 31.4 seconds (1.6 ± 0.7 seconds). There was no significant interaction between factors presented in the model and duration of puppy drinking in the isolation test.

5.3.5 Duration of interaction with ball

The duration that puppies played with the ball varied between 0.0 and 160.0 seconds (9.1 ± 4.0 seconds). Two-way interaction of birth type and anogenital licking on day 6 was associated with duration of time the puppy played with the ball (p=0.011). A one second increase in anogenital licking for caesarean born litters, decreased duration of puppy interaction with the ball (Figure 13).

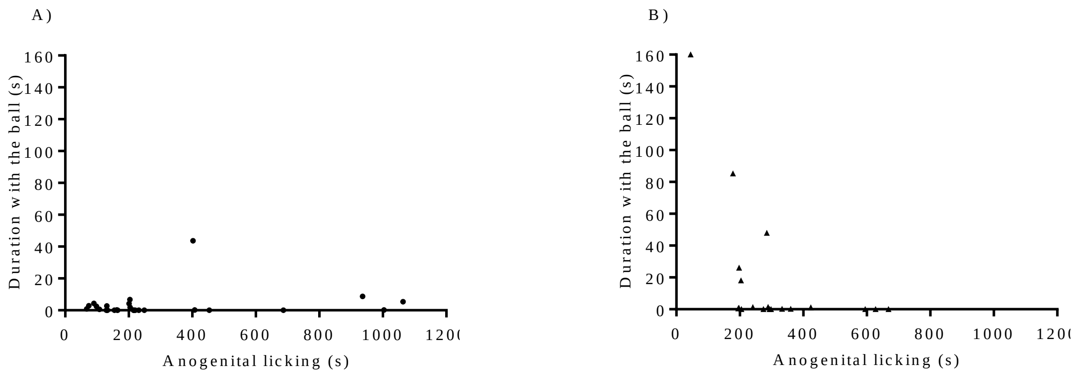


Figure 13: Duration with the ball as a function of the amount of anogenital licking on postnatal day 6 for A) natural and B) caesarean birth types. Each symbol represents an individual puppy in each group.

5.3.6 Latency to vocalise

Latency to vocalise varied between 0.0 and 108.0 seconds (22.9 ± 3.9 seconds). Anogenital licking on day 6 was significantly associated with latency to vocalise (p=0.009). For a 10 second increase in licking, there was a decrease in latency to vocalise of almost half a second (Figure 14). Birth type was marginally significant (p=0.054) for latency to vocalise where puppies born naturally took longer to vocalise (32.4s ± 30.7s, n=28) than caesarean born litters (13.8s ± 18.0s, n=19) (Table 17).

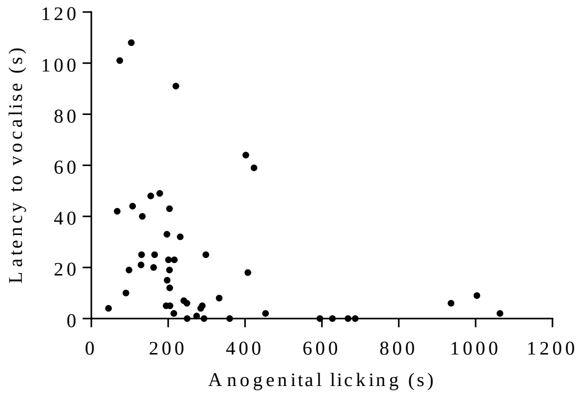


Figure 14: Latency to vocalise as a function of the amount of anogenital licking on postnatal day 6. Each circle represents an individual puppy.

5.3.7 Vocalisation duration

Vocalisation duration varied between 1.7 and 162.7 seconds (109.5 ± 6.7 seconds). There was no significant interaction between factors in the model and duration of puppy vocalisation in the isolation test.

5.3.8 Pre-test heart rate

Pre-test heart rate varied between 120 and 240 bpm (175 ± 3 bpm). Anogenital licking on day 6 was significantly associated with pre-test heart rate (p=0.044). A 10 second increase in anogenital licking, increased the puppy pre-test heart rate by almost half a beat (0.4 bpm).

5.3.9 Post-test heart rate

Post-test heart rate varied between 160 and 264 bpm (202 ± 3 bpm). There was no significant interaction between factors in the model and puppy heart rate after the isolation test.

5.3.10 Difference in heart rate (post - pre)

Difference in heart rate from post- to pre-test varied between -8 and 92 bpm (27 ± 3 bpm). There was a significant association between the two-way interaction of parity and anogenital licking on day 6 and difference in heart rate (post-pre heart rate) (p=0.039). An increase of ten seconds in anogenital licking decreased the heart rate difference by 0.56 bpm in first parity dams (Figure 15).

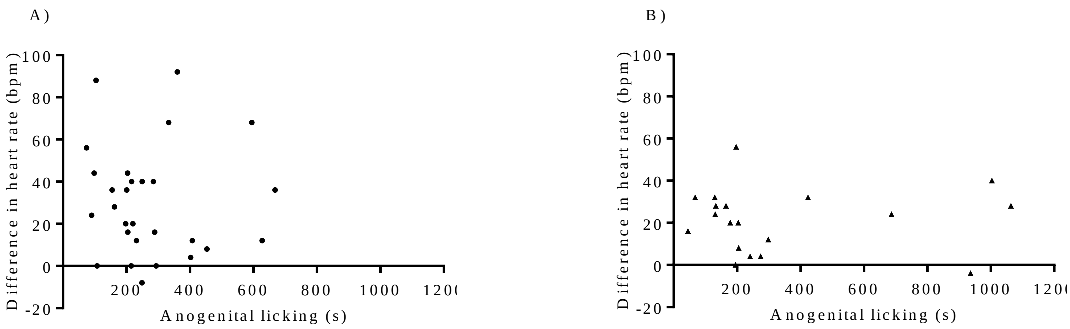


Figure 15: Difference in heart rate as a function of the amount of anogenital licking on postnatal day 6 for A) primiparous and B) multiparous dams. Each symbol represents an individual puppy in each group.

5.3.11 Latency to move post-noise

Latency to move after the bowl was dropped varied between 0.3 and 15.0 seconds (3.0 ± 0.6 seconds). There was no significant interaction between factors in the model and latency to move post-noise.

5.3.12 Latency to vocalise post-noise

Latency to vocalise after the bowl was dropped varied between 1.5 and 30.7 seconds (4.7 ± 0.7 seconds). Two-way interaction of birth type and anogenital licking on day 6 was associated with latency to vocalise post-noise (p=0.035). A 60 second increase in anogenital licking for caesarean born litters increased latency to vocalise by 0.1 second (Figure 16). Puppies born by caesarean took longer to vocalise post-noise (5.0s ± 1.09s, n=19) than puppies born naturally (2.2s ± 0.56s, n=28) (Table 17).

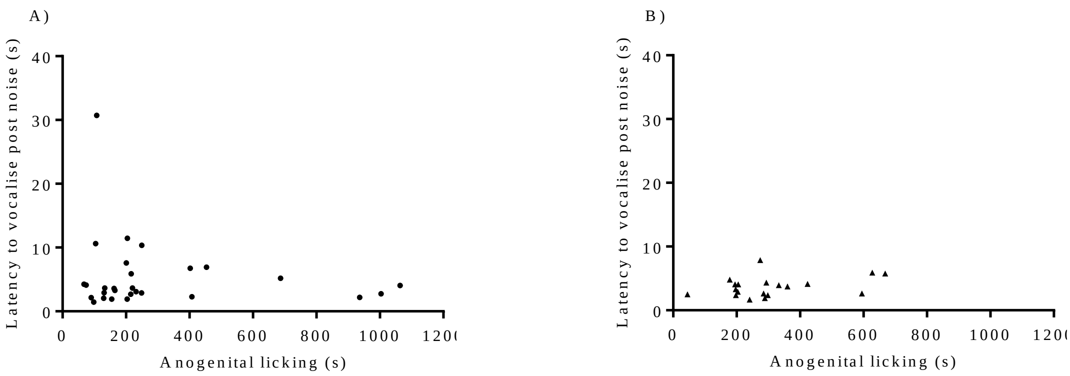


Figure 16: Latency to vocalise post-noise as a function of the amount of anogenital licking on postnatal day 6 for A) natural and B) caesarean birth types. Each symbol represents an individual puppy in each group.

5.3.13 Other factors which may indicate stressful behaviour

Of 47 puppies tested, 19 puppies (40.4%) either urinated or defecated while undertaking the isolation test. Eleven percent (n=5) of puppies both urinated and defecated during the three minutes of observation and these five puppies were from the same litter (Labrador; Litter ID: 10).

5.4 Discussion

This study provides evidence of a link between maternal care and puppy stress response at seven weeks of age. Maternal care and its effect on offspring behaviour was determined using an isolation test. Of note, puppies receiving more anogenital licking on postnatal day 6 vocalised sooner and had a higher pre-test heart rate, suggesting greater stress response. However, puppies licked for a longer duration also responded with a smaller change in heart rate (post – pre heart rate). Overall, puppies receiving more maternal care in the form of anogenital licking showed an increased stress response to isolation, which was opposite to the hypothesis.

A higher level of maternal care may be the foundation of normal puppy development causing the increased stress response observed in those puppies. Onset of fear-related avoidance behaviour in puppies may occur earlier in those licked more during the first 12 postnatal days. Fear-related behaviours are those which are immediate, rapid or pronounced by an individual trying to move away from an object or experience perceived as hazardous (Morrow et al., 2015). Fearfulness of human handlers is apparent when a puppy is five weeks of age, appearing during the socialisation period. However, after two weeks of regular interaction with a human, the fearfulness disappears (Scott and Fuller, 1965). Scott and Fuller (1965) found that the breed of dog does affect onset and ontogeny of fear-related avoidance behaviour. Basenjis were observed running into the corner of the room, crouching and emitting high pitched yelps at five weeks of age which did not occur in other dog breeds (Beagles, Cocker Spaniels, Shelties and Fox Terriers) at the same age. Comparing five dog breeds at seven weeks of age showed a similar response to fear (Scott and Fuller, 1965). Cavalier King Charles Spaniels had a much later onset of fear-related avoidance behaviour than German Shepherds and Yorkshire Terrier puppies (Morrow et al., 2015). Of note, Cavalier King Charles Spaniels that displayed fear-related responses produced a greater cortisol response compared to puppies which did not display the behaviour. The behaviour and morphology of breeds defined as neotonous (an adult appearing with juvenile facial features) also have delayed development, which can also be observed in utero (Trut et al., 2009). This suggests there are differences both between breeds and within breeds in the onset of fear-related avoidance behaviour. Rearing, training and experience also reflected a difference in fearfulness behaviour (Scott and Fuller, 1965; Fox and Stelzner, 1967). Puppies removed from the mother at eight weeks of age and isolated had a much slower approach to a passive observer between 5-8 weeks of age compared with puppies weaned at 31 weeks and reared in complete isolation and puppies weaned at 31 weeks and kept in separate cages (Fox and Stelzner, 1967). Approach behaviour was similar at 12 weeks of age between all groups, suggesting analogous development between animals at 12 weeks (Fox and Stelzner, 1967). The onset of fear-related avoidance behaviour in different dog breeds may be occurring at different times, thus this study may have missed this period in some breeds, affecting the reliability of the results in the current study.

Normal responses to stress may provide a survival advantage to an animal. It is a normal and productive response that when a stressor is presented an acute reaction occurs in the individual. An acute stress response produces a behavioural or/and physiological response allowing the individual to respond to the situation (‘fight or flight’) (Kirby et al., 2013). Acute stress is a single exposure lasting from minutes to hours without repeating re-exposure to the stressor (Kirby et al., 2013). Lupien and McEwen (1997) reported that brief stressors enhance neural function in the brain and memory performance was enhanced in laboratory rats that responded to an acute stressor (Conrad et al., 1999). While acute stress was previously thought to be detrimental to neurogenesis (growth and development of nerve tissue), there is evidence suggesting that acute stress may positively alter growth factors associated with neurogenesis (Mocchetti et al., 1996; Molteni et al., 2001; Kirby et al., 2013). Animals displaying more stressful behaviour in isolation are likely to be reacting positively to an acute stressor (i.e. isolation) and may have different development stages than others. Defecating and urinating can be an indication of stress in dogs (Beerda et al., 1998). Approximately 40% of dogs tested either defecated or urinated during the isolation test, which may highlight stressed behaviour, but may also align with feeding time of the puppies.

The effect of maternal care on puppy behaviour has been documented (Wilsson and Sundgren, 1998b; Foyer et al., 2016; Guardini et al., 2016). Mothers classified as more experienced, as reflected by parity, produced eight-week old puppies with a shorter latency to shriek, increased willingness to play tug-of-war, as well as a decrease in activity and objects visited during the test when observed in the isolation and arena tests (Wilsson and Sundgren, 1998b). This supports the hypothesis above where a response to acute stress may reflect enhanced brain development. Female puppies were observed exploring significantly more and visited more objects in test situations than male puppies (Wilsson and Sundgren, 1998b). This may suggest that sex affects development. However, impact of dam parity/experience may affect puppy behaviour more and this should be investigated first before the impact of puppy sex is determined. In the current study, puppies that were licked more vocalised sooner and were less explorative (as observed by an increased duration in the opening square, fewer lines crossed and reduced interaction with the ball). Guardini et al. (2016) reported eight-week old Beagle behaviour in an isolation test. In contrast to the current study Gazzano et al. (2008) found that puppies born to mothers providing more maternal care were more explorative, had an increased latency to yelp and a decrease in duration of vocalisation and orientation at the enclosure. In the study by Gazzano et al. (2008) maternal care was considered as a combination of contact, licking, anogenital licking and nursing, while the current study only compared anogenital licking to puppy behaviour. It is possible that puppies which seemed more stressed in the current study were more stressed at birth. For example, a stressed animal may vocalise more during the first two postnatal weeks, requiring the mother's attention and hence licked more by the mother. Therefore, it is possible that puppies receiving anogenital licking are more stress-prone, or that stress-prone puppies are seeking more anogenital licking from the dam when young. Although this study did not follow the puppies longer than seven weeks of age, in a previous study, behaviour at 18 months of age was associated with level of maternal care (Foyer et al., 2016). Foyer et al. (2016) found that as maternal care (a combined score of dam in box, lying in contact, nursing, licking and sniff/poke) increased, there was an increase in social and physical engagement, and aggression. It is difficult to compare results of the current study to results presented by Foyer et al. (2016) as a different test was used to determine puppy behaviour and the puppies used in the current study were much younger.

Recovery from a stress response may be shorter in puppies licked more during the first 12 postnatal days. While puppies licked more on postnatal day 6 vocalised sooner and had a higher pre-test heart rate, the change in heart rate from post- to pre-test was smaller in offspring of primiparous dams. Maternal separation and environmental conditions may alter response to stress and recovery period (Francis et al., 2002). Laboratory rats licked more as pups can reduce the length of time they are in an anxious state (Chapter 2 (2.4.1.2)). Receptor density of molecules involved in anti-anxiety effects (benzodiazepine) are higher in offspring of high licking/grooming (LG-ABN) mothers (Caldji et al., 1998). As more binding can occur, an individual can be rendered less anxious for a longer period of time. Also, a receptor involved in mediating the synaptic transmission of nerve terminals and noradrenaline production (α2 adrenoreceptor) has increased numbers in offspring of high LG-ABN litters (Caldji et al., 1998). This produces a reduced response to physiological changes associated with stress, such as a reduced increase in heart rate (Caldji et al., 1998). Hence, puppies may have a stronger behavioural response to stress due to isolation, but have shorter recovery time from the stressful event.

The impact of maternal care and shorter recovery from stress has been reported previously. Rat pups which spent more time with their mother when young had a significantly smaller difference in plasma corticosterone level taken prior to and post-test (Francis et al., 2002). Similarly, in human, infants receiving high quality maternal care had quicker cortisol recovery than infants with lower quality of maternal care (Albers et al., 2008). More crying and a higher cortisol response was observed in infants with more responsive parents. Later, in the infant's first year of life, there was less reactivity to a stressful response and better regulation of the infant (Gunnar et al., 2003). It is likely that the interaction between mother and infant, and postnatal environment, alters stress recovery period.

5.4.1 Isolation box testing

Previous studies (Wilsson and Sundgren, 1998b; Gazzano et al., 2008; Guardini et al., 2016) differed in use of the isolation box. Isolation box dimensions ranged from $1m^2$ (Gazzano et al., 2008) to 2.5m x 3.0m (Wilsson and Sundgren, 1998b). Isolation box size may impede puppy behaviour. Puppies with low exploration duration in a large isolation box may exhibit average to high exploration rate in a smaller isolation box. All studies (Wilsson and Sundgren, 1998b; Gazzano et al., 2008; Guardini et al., 2016) kept the puppy in the box between three and five minutes. As length of time the puppy is separated from the litter increases, the puppy becomes more stressed. Puppies contained in the isolation box for longer will therefore be likely to exhibit increased stress-related behaviours. This may alter results and comparison between studies is difficult due to differing time in isolation. All studies noted vocalisations, while Wilsson and Sundgren (1998b) were the only researchers that did not document activity or exploration behaviour.

Exploration is a critical measure of stress behaviour, in that less exploration and activity usually reflects higher stressor excitement. Gazzano et al. (2008) and the current study included heart rate as a measure of stress behaviour. A physiological measure is advantageous to combine with behavioural measures as, at times, behaviour may not reflect cortisol response (Ramsey and Lewis, 1994; Gunnar et al., 1996). All studies considered here tested puppies aged between seven and eight weeks old, allowing a more reliable comparison to be made. To determine the representativeness of the isolation test for 7-8 week old puppies, further research is needed to determine puppy behaviour during later stages of life. This would allow comparison of the test across a range of ages.

Socialisation of each puppy was not documented for the first seven postnatal weeks in the current study. Puppies may have been exposed to different stimuli and experiences (e.g. crate training), altering reactions in isolation. The same environment should be provided to all puppies to ensure puppy response in isolation is accurately reported. Litter size has been reported to be significantly associated with puppy behaviour (Wilsson and Sundgren, 1998b; Foyer et al., 2013; Foyer et al., 2016) and a larger sample size is necessary to determine the impact of litter size in future studies. There were differences in dog breed used in the current study and it is recommended that the same dog breed or greater litter numbers be used in the future to allow for variation in different breeds.

5.5 Conclusion

In this Chapter, the influence of maternal behaviour during the first two postnatal weeks was observed for puppy behaviour at seven weeks of age. This study provides preliminary evidence that maternal behaviour influences offspring response to stress in isolation. Unexpectedly, puppies receiving higher levels of anogenital licking responded with more stress-related behaviours than those which received a lower duration of licking. As there is evidence that maternal care influences stress response of puppies later in life, maternal care is an important parameter to consider when choosing breeding bitches. Thus, in the next Chapter reasons behind selecting breed stock are described using a survey intended for Australian purebred dog breeders.

Chapter 6. Selection of breeding dams and health practices of Australian purebred dog breeders

Within the previous Chapter, the importance of maternal care and its relationship to the future behaviour and temperament of puppies was discussed. Despite empirical evidence that maternal care plays an important role, it is unclear whether dog breeders are aware, or take this into account when selecting breeding animals and raising puppies. This Chapter seeks to describe the selection of dams by Australian purebred dog breeders.

6.1 Introduction

There are an estimated 4.2 million dogs within Australia (Applied Health Alliance, 2013) with most dogs bred for the purpose of companionship (Bennett and Rohlf, 2007). In Australian, the Australian National Kennel Club (ANKC) is the registered organisation for pedigree dog breeders. In 2015, its 32,481 members, 20% of whom were active breeders, produced (and registered) 69,274 puppies (ANKC, 2015). Given that it is impossible to determine the number of puppies born to non-registered breeders, the total number of puppies born in Australia every year is unknown.

One of the primary aims of the ANKC is to provide members with breed standards that promote behaviourally and physically sound dogs for ownership, as well as promoting excellence in a number of dog related fields, such as breeding, showing, trialling, obedience and other canine related behaviour. Although the ANKC collects information about registered breeders (i.e. number of breeders per breed, number of active breeders, number of litters produced per year, number of puppies produced) through the state bodies, data collected does not extend to the breeding priorities and practices of the breeders.

Such information is vital for improving breeding practices, and ensuring the optimal health and behaviour of dogs.

With over 200 breeds registered with the ANKC, breeding practices are likely to be as diverse as the breeds themselves (Leroy et al., 2007). For example, the purpose for which the dog is bred (i.e. companion, working) is likely to be reflected in the way the dogs are housed and bred. Often, the most important aspect of pedigree or pure breeding involves the selection of breeding animals that conform to a set standard (Pedersen et al., 2013), which is usually determined by a registered organisation such as the ANKC. Physical characteristics (e.g. body conformation, coat length and colour, height, facial appearance, gait), as well as certain behaviours (e.g. instincts such as herding, hunting or retrieving, temperament and trainability) are taken into consideration when choosing breeding stock (King et al., 2009). Priorities of breeders are also likely to alter over time. For instance, in the past, dogs were primarily bred for various working roles, but focus has shifted to selecting for suitable companion animals, shifting towards dog conformation rather than performance (Pedersen et al., 2013). Breed specific diseases are not highly recognised (Patterson et al., 1988; Parker et al., 2004), allowing for accessible knowledge to be implemented by the breeder. Health risks are also being associated with natural mating, and thus sire selection and mating techniques are also necessary to consider (Gill et al., 1970; Thomassen and Farstad, 2009).

To date, the goals and practices of dog breeders across the world have received little attention. Notable exceptions include a study looking at inbreeding and breed effective population size in an Australian sample of breeders (Shariflou et al., 2011), and the selection of dogs and breed goals documented in a French population (Leroy et al., 2007). In that study (Leroy et al., 2007), 985 French dog breeders, representing 10 different breed groups, were asked what considerations they gave to conformation, behaviour, health, work, feeling and reproduction. The behaviour of the dog was considered significantly more important by breeders of sheepdogs, cattle dogs and retrievers compared to all other dog groups. Although the number of litters produced did not significantly alter breed goal, litter production was impacted by breed group; working dogs produced less litters than other breed groups (Leroy et al., 2007). Leroy et al. (2007) also discovered that there were different types of breeders (i.e. occasional, regular hobby and professional breeders) and regular hobby and professional breeders bred from their bitches earlier and therefore had more litters throughout the dam's life. Overall, breeders reported four common goals: (1) dog conformation; (2) behaviour, (3) health and (4) work. Notably, breeders did not consider maternal care as a factor in the selection of breeding bitches (Leroy et al., 2007), despite the importance that it can have on offspring development (e.g., Caldji et al., 1998; Liu et al., 2000; Czerwinski et al., 2016). Other factors including the type of birth (i.e., natural vs caesarean), may also affect the dam's behaviour towards the puppies. Caesareans are more likely to occur in certain breeds according to their cranial features (Jackson, 1995; Linde-Forsberg, 2005; Evans and Adams., 2010), yet there is no literature on the impact of birth type and maternal care.

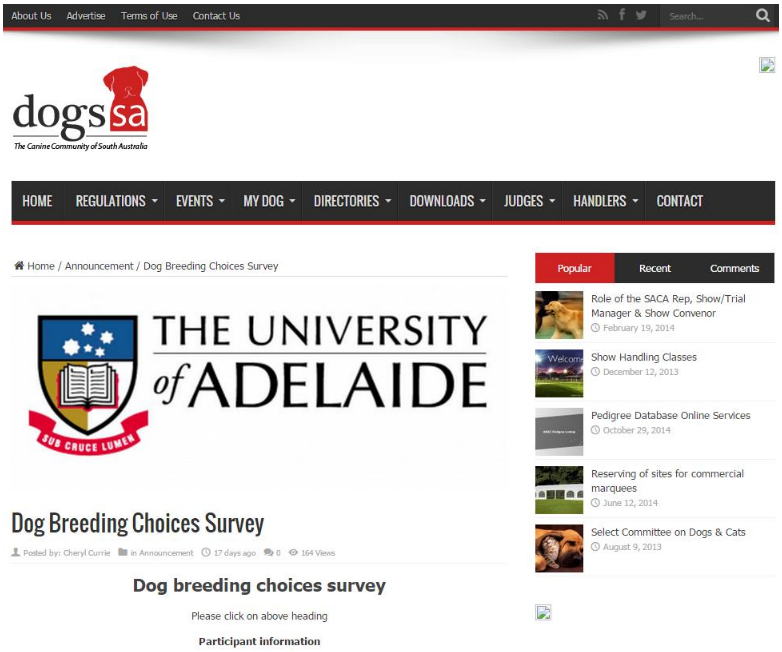
Studies of maternal care behaviour in species such as rats, dogs and humans have shown that maternal care can have implications for the behaviour of young later in life, particularly in relation to their response to stressful events (Caldji et al., 1998; Liu et al., 2000). Maternal care is critical for the survival of altricial animals, where young are born immature (deaf, blind) and rely solely on their mother for survival (Kendrick et al., 1997). However, the level of importance placed on maternal behaviour by dog breeders is unknown (as discussed in Chapter 2). Three recent studies have highlighted the importance of maternal care in dog development (Tiira and Lohi, 2015; Foyer et al., 2016; Guardini et al., 2016). A correlation was found between maternal care and later anxiety in puppies, with poor maternal care in puppyhood increasing the likelihood of anxiety in dogs, measured using questionnaires (Tiira and Lohi, 2015). In the second study, maternal care (dam in box, lying in contact, nursing, licking and sniff/poke) observations were undertaken on 22 litters (Foyer et al., 2016). The dams were observed for the first three postnatal weeks, and then classed as high or low maternal care. By linking maternal care and temperament measured at 15-18 months old, the authors discovered a relationship between the level of maternal care given and physical engagement, social engagement and aggression. An increased interaction between puppy and dam led to adult offspring being more competitive, more engaged in social activities with humans, and higher aggression levels (as defined by the dog's sharpness and defence drive). The amount of maternal care given to the puppies also alters the behaviour of the puppies when they are eight weeks old (Guardini et al., 2016). In a similar study (Guardini et al., 2016) using an isolation test, puppies that were licked more had an increased amount of exploration and a longer latency to first yelp. Increased licking also reduced the duration in locomotion and time spent interacting with the enclosure, and a shorter duration in vocalisation. These data highlight the influence that maternal care can have on future stress responses in puppies.

Currently, information regarding dam and sire selection by Australian dog breeders remains poor. The objective of this study was to understand factors considered important in the selection of Australian purebred breeding animals, with a focus on factors relating to the dam. It was expected that factors such as ANKC breed group, the number of litters produced and, whether the breed is brachycephalic would impact dam selection. The influence of sire selection and health aspects of breeding were also investigated.

6.2 Methods

6.2.1 Survey

A series of questions relating to breeding practices that included questions used by Leroy et al. (2007) was developed. Breeders and ANKC members were then consulted (through email and phone) to ensure that the questions, language and terminology were appropriate for Australian dog breeders. The final survey was hosted on Qualtrics (Qualtrics, LLC) using the URL: http://tinyurl.com/dogbreedersurvey and was available for four months (March to June 2015). The survey was advertised, with permission, on several online resources frequented by dog breeders. These included: Dogs SA (www.dogssa.com.au); Dogs NSW (www.dogsnsw.org.au); public breeder pages on Facebook; Dogz Online forum (www.dolforums.com.au); Vet answers blog (www.vetanswers.com.au/blog); and German Shepherd Club of South Australia (www.gsdcsa.org.au). A description of the survey and the survey link was posted onto the researchers (VC) Facebook page and on an online community noticeboard (www.gumtree.com.au), and the survey was broadcast on local Adelaide radio (101.5 FM).



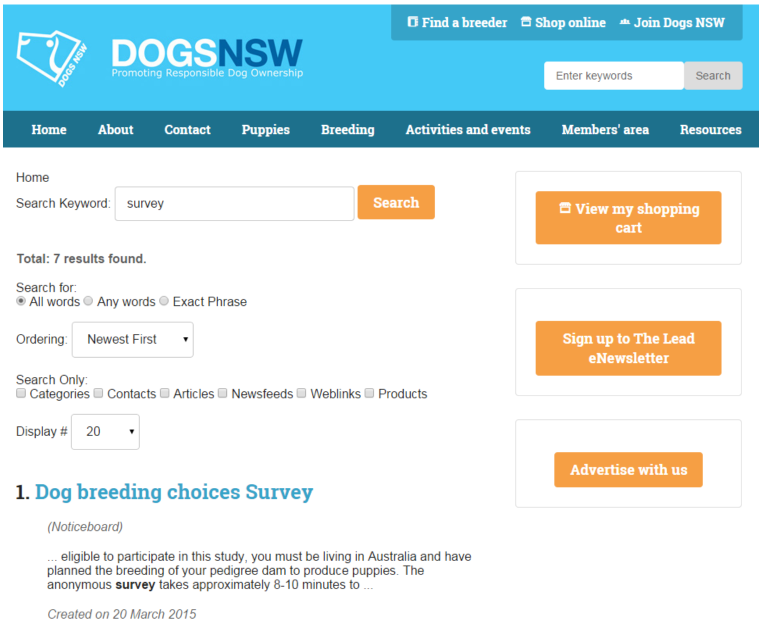


Figure 17a and b: Screenshots from the DOGS SA and DOGS NSW websites, where the dog breeder survey was posted.

The questionnaire was anonymous and participants were not required to respond to all questions. The survey consisted of 58 questions and took approximately 15 minutes to complete. There were four questions pertained to the breeder's demographics; 20 questions related to breeding management; 30 questions related to the importance of qualities associated with the dam and the sire; and four questions relating to DNA and physical testing. A full version of the questionnaire can be found in Appendix 1. The importance of the dam and sire were represented by multiple questions in a Likert scale where the breeder could rate the importance from Strongly Agree (1) to Strongly Disagree (5). All responses were considered for the first breed listed by the dog breeder, if the breeder bred more than one dog breed. Approval from the University of Adelaide Human Ethics Committee was obtained (H-2014-270).

6.2.2 Statistical Analysis

6.2.2.1 Data transformation

For open-ended responses where a range was given as a response, the average of the range was used. For example, when asked "On average, how often will you breed from each bitch?" an answer of '1-3' was then changed to '2'. To describe the physical and genetic tests performed, only those breeds with more than five respondents were included. This allowed satisfactory comparison within the data for types of dog testing. Breeders that bred more than one dog breed were excluded to remove any confusion as to which DNA or physical test was undertaken on which breed. Although some physical and genetic tests are breed specific, many times the response could be applied to several breeds (i.e. x-ray for hip score).

6.2.2.2 Univariate analysis of variance (ANOVA)

Normality was not achieved in the components (components were positively skewed) and therefore they were log transformed. The Tukey method (Mosteller and Tukey, 1977) was used to determine outliers. Firstly, the first and third quartile were identified. The Interquartile range (third quartile minus the first quartile) was then multiplied by 1.5. This value was subtracted from the first quartile and again added to the third quartile. Numbers which fell below these values were identified as outliers. Three breeders were removed as they were determined as outliers (Case numbers: 69, 180 and 223), resulting in 271 breeders for results using ANOVA. Twenty-three items relating to the dam were reduced into five components using Principal Component Analysis. However, after observing the matrix, one component was removed leaving four components. Components were analysed using a univariate general model to determine whether there were differences between Australian National Kennel Club (ANKC) dog breed group, number of litters produced, number of breeds the breeder owns and breeds, and brachycephalic dog breed. An eta-squared value (η^2) was calculated; this refers to the effect size (strength between two variables) and is described for each ANOVA. Tukey post-hoc tests were used to determine significance between pairwise comparisons of means. All statistical analyses were performed using SPSS (IBM, v.21). Significance was accepted at $p<0.05$.

6.3 Results

6.3.1 Participants

A total of 360 Australian purebred dog breeders completed the survey. However, 86 participants (24%) were discarded due to insufficient responses resulting in a total of 274 (unless stated otherwise). In particular, if questions regarding the dog breed or dam behaviour were not answered, the respondent's results were removed. A total of 91 dog breeds were represented in the survey, and are described in Table 18. The majority of participants bred Working dogs (21.2%) followed by Gundogs (19.3%), while the Toy group (9.9%) were the least represented (Table 18).

Brachycephalic dog breeds are those which have a facial skeleton relatively shorter than the cranial cavity (Evans and Adams, 2010). These breeds included: Australian Silky Terrier, Boston Terrier, Boxer, British Bulldog, Bullmastiff, Cavalier King Charles Spaniel, Chihuahua, Dogue de Bordeaux, French Bulldog, Havanese, Papillon, Pug, Rottweiler, Shar Pei, Shih Tzu, Staffordshire Bull Terrier and Tibetan Spaniel (Koch et al., 2003; Leroy et al., 2005; Torrez, 2006; Gacsi et al., 2009; Carrasco et al., 2014).

Table 18: Dog breeds represented in the survey

Group 1: Toys	N	%	Group 2: Terriers	N	%	Group 3: Gundogs	N	%	Group 4: Hounds	N	%
Australian Silky Terrier*	2	0.7	American Staffordshire Terrier	1	0.4	Brittany	1	0.4	Afghan Hound	2	0.7
Cavalier King Charles Spaniel*	4	1.5	Bull Terrier	2	0.7	Cocker Spaniel	8	2.9	Basenji	5	1.8
Chihuahua*	1	0.4	Bull Terrier Miniature	1	0.4	Field Spaniel	2	0.7	Beagle	3	1.1
Chinese Crested Dog	1	0.4	Jack Russell Terrier	2	0.7	Flat Coated Retriever	1	0.4	Borzoi	1	0.4
Havanese*	2	0.7	Scottish Terrier	2	0.7	German Shorthaired Pointer	5	1.8	Dachshund (Min. Long)	1	0.4
Italian Greyhound	6	2.2	Soft Coated Wheaten Terrier	1	0.4	Golden Retriever	15	5.5	Dachshund (Min. Smooth)	1	0.4
Miniature Pinscher	2	0.7	St. Bernard	1	0.4	Gordon Setter	1	0.4	Deerhound	1	0.4
Papillon*	2	0.7	Staffordshire Bull Terrier*	17	6.2	Hungarian Vizsla	1	0.4	Petit Basset Griffon Vendeen	1	0.4
Pug*	5	1.8	Tenterfield Terrier	1	0.4	Hungarian Wirehaired Vizsla	1	0.4	Rhodesian Ridgeback	3	1.1
Tibetan Spaniel*	2	0.7	West Highland White Terrier	5	1.8	Labrador Retriever	12	4.4	Saluki	4	1.5
						Nova Scotia Duck Trolling Retriever	3	1.1	Whippet	6	2.2
						Weimaraner	2	0.7			
						Welsh Springer Spaniel	1	0.4			
Total Group 1	27	9.9	Total Group 2	33	12.0	Total Group 3	53	19.3	Total Group 4	28	10.2

*Brachycephalic dog breed.

Table 18 continued

Group 5: Working dogs	N	%	Group 6: Utility	N	%	Group 7: Non-Sporting	N	%
Australian Cattle Dog	1	0.4	Alaskan Malamute	3	1.1	Boston Terrier*	1	0.4
Australian Kelpie	6	2.2	Boxer	6	2.2	British Bulldog	2	0.7
Australian Shepherd	6	2.2	Bullmastiff	2	0.7	Dalmatian	1	0.4
Belgian Shepherd Dog	7	2.6	Cane Corso	2	0.7	French Bulldog*	1	0.4
Border Collie	13		Dobermann	2	0.7	German Spitz	1	0.4
Collie (Rough)	2	0.7	Dogue de Bordeaux*	1	0.4	Great Dane	7	2.6
Collie (Smooth)	1	0.4	German Pinscher	1	0.4	Japanese Spitz	1	0.4
Finnish Lapphund	2	0.7	Leonberger	1	0.4	Poodle (Miniature)	3	1.1
German Shepherd Dog	13	4.7	Neapolitan Mastiff	1	0.4	Poodle (Standard)	7	2.6
Maremma Sheepdog	1	0.4	Newfoundland	2	0.7	Poodle (Toy)	2	0.7
Shetland Sheepdog	2	0.7	Old English Sheepdog	2	0.7	Schipperke	1	0.4
Welsh Corgi (Cardigan)	1	0.4	Portugese Water Dog	1	0.4	Shar Pei*	1	0.4
Welsh Corgi (Pembroke)	3	1.1	Pyrenean Mountain Dog	1	0.4	Shih Tzu*	1	0.4
			Rottweiler*	10	3.6	Tibetan Terrier	1	0.4
			Russian Black Terrier	2	0.7	Xoloitzcuintle	2	0.7
			Samoyed	1	0.4			
			Schnauzer	1	0.4			
			Siberian Husky	3	1.1			
			Tibetan Mastiff	1	0.4			
Total Group 5	58	21.2	Total Group 6	43	15.7	Total Group 7	32	11.7

*Brachycephalic dog breed (Brachycephalic dog breeds are those which have a facial skeleton relatively shorter than the cranial cavity (Evans and Adams, 2010).

6.3.2 Principal components analysis

The 23 items relating to the dam were reduced using principal components analysis (PCA). Prior to performing PCA, the appropriateness of the data for analysis was assessed. The correlation matrix revealed the presence of many coefficients of 0.30 and above, the Kaiser-Meyer-Olkin exceeded the recommended value of 0.60 at 0.87, and Bartlett's Test of Sphericity reached statistical significance, supporting the factorability of the correlation matrix. Five components were presented with an eigenvalue exceeding 1, explaining 64.06% of the total variance. After observing the rotated component matrix, four questions were removed due to either a component loading lower than 0.55 ("good cut off loading" (i.e. 'my dam is confident' and 'my dam has an outstanding pedigree') or too few items within the component (i.e. component 5 contained two items: 'my dam has outstanding conformation according to the breed standard' and 'my dam is within the accepted size according to the breed standard') (Tabachnick and Fidell, 2007). This resulted in 19 items being retained (Table 19) and four components remaining which explained 63.2% of the total variance.

Table 19: Loading for the 19 items of the dam scales generated by Principal Component Analysis

	Component			
Survey question	**1**	**2**	**3**	**4**
My dam has naturally conceived with ease	0.733			
My dam has whelped with ease	0.803			
My dam has an excellent maternal instinct towards her puppies	0.729			
My dam produces sufficient milk to raise her puppies	0.762			
I would not breed from a dam if conception and whelping were difficult	0.675			
I would not breed from a dam if her maternal behaviour was not ideal	0.625			
I would not breed from a dam if her puppies did not conform closely to the breed standard		0.705		
I would not breed from a dam if the temperament of her puppies was not ideal		0.761		
I would not breed from a dam if she had rejected her puppies		0.567		
I would not breed from a dam if some of her puppies had a significant genetic fault		0.735		
I would not breed from a dam that was aggressive towards unknown people		0.605		
My dam is excitable			0.617	
My dam is obedient			0.806	
My dam has a strong bond to humans			0.659	
My dam is trainable			0.761	
My dam has an optimal temperament for the breed				0.747
My dam has passed all required and recommended health tests for her breed				0.735
My dam comes from a line of healthy, long lived relatives				0.658
My dam is friendly to dogs, other animals and people				0.604

The four components were labelled: Maternal Care (Component 1), Offspring Potential (Component 2), Dam Temperament (Component 3) and Dam Genetics and Health (Component 4). Component 1 was labelled Maternal Care as all questions within this component related to conception, whelping and the dams ability to raise and care for her puppies. Offspring Potential was labelled for Component 2 as the questions related to the offspring's look and temperament. This Component also included the potential for temperament and genetic traits to be passed on to future generations. For example, if the dam is aggressive or rejects her puppies it may be likely that her offspring will do the same. Dam Temperament was considered for Component 3 as all questions within this component related to the dams behaviour. Component 4 was labelled Dam Genetics and Health as questions within this component related to health and genetically driven behaviours. The lower the score, the more the respondents found the component to be important. There were six questions related to maternal care with a range of 6-30; five questions related to Offspring Potential and a range of 5-25; and both genetics/health and temperament contained four questions each with a range from 4-20 for each component. Cronbach's alpha for these components were: 0.861, 0.747, 0.741, and 0.718 respectively.

6.3.3 General characteristics of the breeders

The majority of respondents were females (88%) and were aged between 18 and 85 years (n=270: mean=50.6, SD=13.4). Most of the participants lived in New South Wales (41.6%) followed by South Australia (24.1%), Queensland (11.7%), Victoria (9.1%), Western Australia (8.8%) and Tasmania (2.6%). Six respondents (2.2%) did not report the state they were from. Half of the respondents had completed either high school (25.9%) or Technical and Further Education (TAFE) (25.9%). Some participants had completed an undergraduate degree (16.4%) with another 72 respondents (26.3%) completing a postgraduate degree. Of the remaining respondents, 5.1% had completed something other than that described, such as a diploma or a trade.

The most common dog breed within the survey was the Staffordshire Bull Terrier (n=17, 6.2%). Participants were most likely to breed only one breed of dog (76.6%), with some participants breeding two (19.3%) or three breeds (4.0%). Around one third (33.5%) had been breeding between 0-9 years, with the remaining having bred more than 10 years (Table 20). Most breeders owned dams and dogs (80.3%), however 39 breeders (14.2%) only owned bitches, and 11 breeders (4.0%) only owned dogs. The remaining breeders (n=4) owned neither dogs nor bitches, hence were former breeders. Just under 53% of breeders bred a bitch 2 times or less, while 42% of breeders bred their dams between 2 and 5 times. A small number of breeders (5.5%, n=15) bred their dams more than 5 times and a maximum of 10 times in the dams life. Almost all respondents (n=267 or 97.4%) were part of the ANKC. Of the five respondents (1.8%) which were not part of the ANKC, one breeder was associated with a Working dog association recognised by the ANKC and another was a member of a breed group/club.

Table 20: Number of years dog breeders had bred and owned their dogs

	No. of years breeding dog		No. of years owning dog	
Years	*N*	%	*N*	%
<5 years	59	21.5	13	4.7
5-9 years	33	12.0	38	13.9
10-19 years	65	23.7	73	26.6
20-29 years	51	18.6	70	25.5
30-39 years	32	11.7	53	19.3
40+ years	33	12.0	27	9.9

6.3.4 Dam breeding priorities and ANKC breed group

The importance of maternal care differed significantly between ANKC breed groups (F (6, 264) = 2.41, p = 0.028; partial η^2 = 0.05). The Toy and Hound dog breeding groups scored Maternal Care significantly more important than the Terriers, Gundogs, Working dogs and Utility groups. The Non-sporting dog breeding group scored maternal care significantly more important than the Utility breeds. Other components were not significant for breed group: Offspring Potential (F (6, 264) = 1.69, p = 0.123; partial η^2 = 0.04), Dam Temperament (F (6, 264) = 1.14, p = 0.341; partial η^2 = 0.03) and Dam Genetics and Health (F (6, 264) = 1.59, p = 0.150; partial η^2 = 0.04) (Table 21).

Table 21: Principal Component Analysis subscale relating to the Dam according to ANKC breed group. Lower values represent higher importance

Outcome Variable	df	*Df error*	*F*	Breed group	Means	95% Confidence Interval Lower bound	Upper bound
Maternal care	6	911.73	2.408	Toys	0.916	0.858	0.974
				Terriers	0.986	0.934	1.038
				Gundogs	0.977	0.937	1.018
				Hounds	0.906	0.850	0.962
				Working dogs	1.000	0.962	1.038
				Utility	1.011	0.965	1.057
				Non Sporting	0.959	0.907	1.011
Offspring Potential	6	911.73	1.693	Toys	0.818	0.767	0.868
				Terriers	0.860	0.814	0.905
				Gundogs	0.855	0.820	0.891
				Hounds	0.827	0.778	0.875
				Working dogs	0.893	0.859	0.926
				Utility	0.845	0.805	0.885
				Non Sporting	0.825	0.780	0.871
Dam Temperament	6	911.73	1.138	Toys	0.913	0.857	0.969
				Terriers	0.929	0.878	0.979
				Gundogs	0.923	0.884	0.962
				Hounds	0.993	0.939	1.047
				Working dogs	0.937	0.899	0.974
				Utility	0.964	0.919	1.008
				Non Sporting	0.934	0.883	0.984
Dam Genetics & health	6	911.73	1.591	Toys	0.674	0.631	0.718
				Terriers	0.702	0.663	0.742
				Gundogs	0.678	0.647	0.708
				Hounds	0.747	0.705	0.789
				Working dogs	0.704	0.675	0.733
				Utility	0.691	0.657	0.726
				Non Sporting	0.675	0.636	0.715

6.3.5 Breeding priorities relating to the Dam and the number of litters produced

Two hundred and forty-two (89.3%) breeders bred two litters or less a year, while 29 breeders (10.7%) bred more than two litters per year. Components were not significantly different compared to the number of litters produced per year: Maternal Care (F (1, 267) = 3.09, p = 0.080; partial η^2 = 0.07), Offspring Potential (F (1, 267) = 0.02, p = 0.892; partial η^2 = <0.01), Dam Temperament (F (1, 267) = 0.05, p = 0.817; partial η^2 = <0.01) and Dam Genetics and Health (F (1, 267) = 2.02, p = 0.156; partial η^2 = 0.03).

6.3.6 Breeding priorities in relation to the Dam and the number of dog breeds

Seventy-six percent of breeders bred one dog breed (n=207). Dam Temperament was significantly affected by the quantity of breed types that the breeder bred (F (2, 268) = 3.17, p = 0.044; partial η^2 = 0.07) (Table 22). The breeders of one breed type placed more importance on Dam Temperament compared to breeders which bred two types of breeds. There were no significant differences between breed number and the other components: Maternal Care (F (2, 268) = 1.15, p = 0.317; partial η^2 = 0.03), Offspring Potential (F (2, 268) = 2.36, p = 0.097; partial η^2 = 0.04), and Dam Genetics and Health (F (2, 268) = 0.91, p = 0.403; partial η^2 = 0.01).

Table 22: Breed priority and the number of breeds that the participant bred

						95% Interval	Confidence
Outcome Variable	df	*Df error*	*F*	Number of breeds	Means	Lower bound	Upper bound
Maternal care	2	530	1.154	1	0.965	0.945	0.986
				2	1.000	0.959	1.042
				3	0.988	0.894	1.083
Offspring Potential	2	530	2.357	1	0.847	0.829	0.864
				2	0.859	0.823	0.894
				3	0.937	0.856	1.018
Dam Temperament	2	530	3.168	1	0.929	0.909	0.949
				2	0.985	0.946	1.024
				3	0.946	0.857	1.036
Dam Genetics & health	2	530	0.911	1	0.691	0.675	0.706
				2	0.704	0.673	0.735
				3	0.734	0.664	0.805

6.3.7 Breeding priorities relating to the Dam and brachycephalic dog breeds

There were 54 breeders (19.9%) with brachycephalic dogs. Offspring Potential (F (1, 269) = 5.14, p = 0.024; partial η^2 = 0.09) and Dam Genetics and Health (F (1, 269) = 4.33, p = 0.038; partial η^2 = 0.06) significantly differed when comparing whether the dog was brachycephalic or not. Breeders of brachycephalic dogs scored Offspring Potential and Dam Genetics and Health significantly more important than breeders of non-brachycephalic dogs. There were no significant difference for non-brachycephalic and brachycephalic breeds in Maternal Care (*F* (1, 269) = 0.09, *p* = 0.771; partial $\eta^2 < 0.01$), and Dam Temperament (*F* (1, 269) = 1.51, *p* = 0.221; partial $\eta^2 = 0.03$) (Table 23). Seven breeders bred at least two brachycephalic dog breeds (2.6%) while 26 of the 63 breeders breeding more than one breed type bred at least one brachycephalic dog breed (9.5%).

Table 23: Components according to whether the dog breed was brachycephalic

						95% Confidence Interval	
Outcome Variable	df	*Df error*	*F*	Brachycephalic breed	Means	Lower bound	Upper bound
Maternal care	1	266	0.085	No	0.974	0.954	0.995
				Yes	0.968	0.927	1.008
Offspring Potenial	1	266	5.138	No	0.861	0.844	0.879
				Yes	0.816	0.781	0.851
Dam Temperament	1	266	1.505	No	0.946	0.926	0.965
				Yes	0.919	0.880	0.958
Dam Genetics & health	1	266	4.326	No	0.702	0.687	0.717
				Yes	0.666	0.636	0.697

6.3.8 Sire selection

Twenty-nine percent of breeders accessed a distant sire owned by someone else, 23.7% of breeders used their own sire, while accessing a local sire for breeding was less common (9.9%). For breeding, the breeders own sire or a local sire was most commonly used (35.4%), however others imported frozen semen (6.2%) or imported the sire (0.7%). The number of breeders opting to use artificial insemination was rather small in the dataset (7.3%). Many breeders (67.8%) spent time interacting with the sire before selection but 28.0% of breeders found it not possible. Variables affecting sire selection are displayed in Table 24. Breeders rated the sire's conformation and temperament highly, together with his ability to produce healthy puppies and complementing the dam.

Table 24: Variables important to the selection of the sire

Variable Importance	Location	Conformation	Size	Pedigree	Temperament
High	6.0	95.5	84.7	74.3	98.9
Neutral	10.9	3.7	14.6	20.5	1.1
Low	83.0	0.7	0.7	5.2	0.0

Table 24 continued

	Respondents (%)	
Variable Importance	**Complements dam**	**Healthy puppies**
High	99.6	95.5
Neutral	0.0	3.7
Low	0.4	0.7

6.3.9 Physical and genetic testing of both Dams and Sires

If more than five respondents represented a single breed, they were identified for physical and genetic tests conducted. This occurred for 11 dog breeds (Table 25). Some breeders undertook more tests than others and many of the breeders undertook tests specific to their breed. For example DNA testing for Neuronal Ceroid Lipfuscinosis (NCL) was undertaken by all 12 Border Collie breeders while 11 out of the 14 Golden Retriever breeders undertook hip scoring.

Table 25: Genetic and Physical testing of common dog breeds within the survey

Breed	Breeders (n)	DNA test	Breeders undertaking test (n)	Physical tests	Breeders undertaking test (n)
Basenji	5	Fanconi	5	Eye assessment	5
		Progressive Retinal Atrophy	4	Hip score	2
		Hemolytic anaemia	2	Thyroid	2
		Pyruvate kinase deficiency	1	Heart assessment	1
		DNA inbreeding coefficient Factor 7	1		
		DNA identification	1		
		Thyroid			
Belgian Shepherd Dog	5	Colour MDR1 masking	1	Hip score	3
				Elbow score	*3*
				Eye assessment	*2*
				Heart assessment	*1*
Border Collie	12	Neuronal Ceroid Lipofuscinosis	12	Elbow score	11
		Trapped Neutrophil Syndrome	11	Hip score	10
		Collie Eye Anomaly	10	Eye assessment	6
		Multi-Drug Resistance Gene 1	2	General vet check	1
		Imerslund-Grasbeck Syndrome	2	Chiropractor vet check	1
		Degenerative Myelopathy	1	Collie collapse	1
		Parentage (Orivet)	1	Hearing test	1
		Glaucoma	1		
		B12	1		

Table 25 continued

Breed	Breeders (n)	DNA test	Breeders undertaking test (n)	Physical tests	Breeders undertaking test (n)
German Shepherd Dog	11	Degenerative Myelopathy	6	Hip score	6
		Ivermectin Sensitivity	2	Elbow score	6
		Long stock coat gene	1	X-ray (not specified)	1
		Canine renal Dysplasia	1	Vet check	1
		Dwarfism	1		
		Haemophilia	1		
Golden Retriever	14	Ichthyosis	12	Hip score	11
		Progressive Retinal Atrophy 1	10	Eye assessment	11
		Progressive Retinal Atrophy 2	10	Heart assessment	11
		Progressive Rod Cone Degeneration	4	Elbow score	10
				Dentition assessment	1
Great Dane	5	Heart testing	5	Hip score	5
		Thyroid	4	Elbow score	5
		Colour DNA	1	Shoulder and neck x-rays	1
Labrador Retriever	11	Progressive Retinal Atrophy	10	Hip score	5
		Exercise-induced Collapse	8	Elbow score	5
		Progressive Rod Cone Degeneration	2		
		Coat colour	1		
		Long hair	1		
		DNA identification	1		
		Centronuclear Myopathy	1		

Table 25 continued

Breed	Breeders (n)	DNA test	Breeders undertaking test (n)	Physical tests	Breeders undertaking test (n)
Poodle (Standard)	5	Degenerative Myelopathy	3	Eye assessment	4
		Neonatal Encephalopathy	3	Hip score	4
		von Willebrand's disease	3	Skin biopsy	1
		Thyroid	2	Vet checked	1
		Full DNA data	1		
		Progressive Retinal Atrophy	1		
		Renal Dysplasia	1		
Rottweiler	8	DNA testing	1	Hip score	8
		von Willebrand's disease	1	Eye assessment	8
				Dentition assessment	8
				Elbow score	7
				Heart assessment	3
				Joint assessment	1
				Brachycephalic Obstructive Airway Syndrome	1
Staffordshire Bull Terrier	16	Hereditary Cataracts	12	Eye assessment	7
		L2-Hydroxyglutaric aciduria	12	Hip score	3
		Full DNA test	2	Heart assessment	2
		Coat colour	1	Elbow score	1
				Dentition assessment	1
				X-ray (not specified)	1
				Vet check	1
West Highland White Terrier	5	Genetic technologies	1		

6.4 Discussion

This study aimed to gain insight into breeding stock selection of Australian purebred dog breeders, with particular emphasis on dams. An overview of the general characteristics and breeding priorities of a small sample of Australian purebred dog breeders covering 91 different breeds across seven breed groups classified by the ANKC. The majority of 274 breeder's surveyed bred only one dog breed, kept three and two bitches respectively, and bred two litters or less a year. The implications of this study include the potential to provide the findings to dog breeding groups and governing bodies which may endorse important breeding priorities and thus produce improved dog litters.

6.4.1. The Impact of the Number of Litters Produced and the Number of Dog Breeds on Breed Priority Relating to Dams

In our sample of active and non-active breeders, almost three quarters (69%) produced one litter or less per year, which is slightly above those presented by the ANKC (54%) (ANKC, 2015). However, we found fewer breeders breeding 5–10 litters per year (2% compared to 5% respectively). Consistent with Leroy et al. (2007) in a population of French breeders, we found that the number of litters produced was not significantly associated with breed priority. It appears that the larger kennels (determined by the number of breeding bitches and litters produced) within the current study are observant of their breeding dogs, and prioritise the health and wellbeing of their animals to a similar extent to smaller kennels. We did however, find that the breed related to the priorities and practices of the breeder. For instance, breeders of hunting and working dogs produce less stock as they are breeding to satisfy their own needs and replenish their working stock (Leroy et al., 2007). While breeders of working stock may be breeding for their own purpose, breeders of increasingly popular dog breeds, such as the Pug (Asher et al., 2009), may be producing stock for companionship and therefore litters produced would be higher.

It was expected that Dam Temperament would feature as a component from the principal component analysis given that there is a large body of work investigating temperament in dogs (e.g., Goodloe and Borchelt, 1998; Jones and Gosling, 2005; Starling et al., 2013). Unlike the number of litters produced per year, breeders that bred a single breed rated Dam Temperament as significantly more important than breeders that bred more than one breed. Statements in this component included the dam being excitable, obedient, having a strong bond to humans, and trainable. A possible reason for the differences observed between the breeds may be due to the different dog breeds which vary in their levels of excitement, obedience and trainability. Thus, the breeder may not necessarily share the same focus across multiple breeds, which would then reduce the priority of Dam Temperament. Research into the impact of the number of breeds kept and the impact on Dam (as well as sire) Temperament is currently lacking, and needs to be investigated further.

6.4.2. Breeder Priorities in Relation to Maternal Care

Of the four breed priorities relating specifically to dams identified through PCA, questions relating to breeding and dam-puppy interactions were identified. Studies of maternal care behaviour in species such as rats, dogs and humans have shown that maternal care can have implications for the behaviour of young later in life, particularly in relation to their response to stressful events (Caldji et al., 1998; Liu et al., 2000; Czerwinski et al., 2016). Maternal care is critical for the survival of altricial animals, where young are born immature (deaf, blind) and rely solely on their mother for survival (Kendrick et al., 1997; Czerwinski et al., 2016). Priorities relating to Maternal Care significantly differed between ANKC dog breed group, suggesting that it might be more relevant for some, but not all breeds. For instance, Maternal Care was a higher priority for breeders of Toy and Hound ANKC groups compared to the Terrier, Gundog, Working dog and Utility groups. The Maternal Care component was a mixture of statements, including conceiving and whelping with ease, as well as maternal instinct and milk production.

The majority of brachycephalic dogs consisted of breeds from the Toys and Non-sporting ANKC groups. It is common for brachycephalic dams to experience dystocia (Jackson, 1995; Linde-Forsberg, 2005; Evans and Adams., 2010), difficulty of birthing the puppies naturally through the birth canal (Forsberg and Persson, 2007; Asher et al., 2009), and forcing the dam to have a caesarean birth. Bitches requiring caesareans due to dystocia account for more than 60% of births (Gaudet, 1985; Jackson, 1995; Bergstrom et al., 2006). Maternal care may be impaired in dams recovering from surgery due to a caesarean birth, causing the breeder to be more involved with the litter. The dam is needed within the litter for puppy survival and development. The puppies not only feed from their mother when very young, but are also influenced by her temperament and behaviour. Further understanding of breed priorities according to the brachycephalic index would allow targeted implementation of breed standards and criteria. While comparative data on conception and prevalence of caesarean sections is lacking across all dog breeds, a recent study highlighted a general reduction in the fertility of male dogs (Lea et al., 2016). In a retrospective cohort study of Norwegian purebred dogs, 8% of puppies died before eight days of age, in 10,810 litters and 224 breeds (Tonnessen et al., 2012). As well as the age of the bitch and litter size, breed was also an important factor influencing perinatal mortality, although the largest variation was between litters. In breeds that experience problems in conception, birthing type of perinatal mortality, it is likely that the Maternal Care component would score more highly. For example, lack of maternal instincts may mean breeders have to work much harder to keep the puppies alive, and if early colostrum is not received by the puppies, then they will be less likely to thrive (Mila et al., 2015). We recommend that future studies focus on questions relating specifically to maternal care and birthing method to determine the true influences on breeders' choices in this area. An example of an important initiative in this area is the veterinary reporting of caesareans and procedures to alter the natural conformation of dogs being supported in the UK by the Kennel Club, British Veterinary Association, British Small Animal Veterinary Association and Royal College of Veterinary Surgeons (Kennel Club, 2015). Of course, we acknowledge that it is not possible to select for dam maternal behaviour until it has been observed at least once. So, although it will not influence the initial decision to breed a bitch, it should be considered for subsequent breeding.

6.4.3. Breeder Priorities in Relation to Genetics and Health

There are a large number of hereditary diseases identified in the dog population, second to humans (Brooks and Sargan, 2001). This allows dog diseases to be identified and possibly treated in several breeds. Over 350 inherited disorders have been categorised by the American Kennel Club (Patterson et al., 1988). Of these diseases, many are restricted to specific breed groups or particular breeds (Parker et al., 2004), such as syringomyelia in the Cavalier King Charles Spaniel (Parker et al., 2004). Some diseases affect a large majority of dog breeds, e.g., hip and elbow dysplasia (Fries and Remedioa, 1995; Wooliams et al., 2011; Hou et al., 2013), and their heritability and incidence are continually being revised and reported. Reflective of this, breeders surveyed in this study rated Dam Genetics and Health as the most important component when selecting dams, and actively conducted genetic and physical testing of their dogs. Brachycephalic dog breeders gave even higher importance to Dam Genetics and Health than breeders of non-brachycephalic dog breeds. It is assumed that breeders of brachycephalic dogs are aware of the problems associated with these breeds of dogs (e.g., obstructive airway syndrome (BOAS) is a common constraint for brachycephalic breeds (Packer et al., 2012)), and therefore place more importance on additional genetic and health aspects which may also be present. Genetic testing was more relevant to some breeds than others, likely reflecting known issues within the breed. For example, all of the Rottweiler breeders assessed their dogs for dentition assessment, indicating that this is an important problem for the breed. Eight breeds were assessed for elbow scoring. Indeed, elbow dysplasia and borderline signs were observed in half of the Rottweilers included in an official screening program (see Heine et al., 2008).

Important diseases have been recorded in dogs and by all breeders (http://discoveryspace.upei.ca/cidd/breeds/overview). A review of common disorders inherited in purebred dogs is also available (Tiira and Lohi, 2015). In purebred dogs, in a case-control study for 24 common hereditary disorders, 10 disorders were found to be more prevalent than in mixed-breed dogs, suggesting a greater proportion of diseases occurring in purebred than in mixed-breed dogs (Bellumori et al., 2013). Multiple disorders are associated with brachycephalic breeds: BOAS in Boston Terriers and Pugs (Lorinson et al., 1997), dystocia in Bull Terriers (Asher et al., 2009), eye problems in Pugs and Shar Peis, and mitral valve disease in Cavalier King Charles Spaniels (Asher et al., 2009). Only one breeder of six described undertaking a breathing test for their pug, although 67% (n = 6) documented eye testing. While no common tests were undertaken by Boston Terrier breeders (n = 2) and Shar Pei breeders (n = 1), almost all breeders of Cavalier King Charles Spaniels (6/7) had hear auscultations as a physical test undertaken on their dogs. It seems important to highlight that a common disease test for the Pug is not being undertaken, and needs further investigation as to the reasons behind this. Although Dam Genetics and Health were deemed more important by brachycephalic breeders, it seems that they are not always undertaking the relevant tests. In the current dataset, all Great Dane breeders tested for dilated cardiomyopathy and almost all of Rottweiler breeders tested for elbow dysplasia. The top ten significant disorders of the ANKC include hip dysplasia, epilepsy, hypothyroidism, allergies, hemangiosarcoma, patella luxation, cataracts, lymphoma, bloat and progressive retinal atrophy (Bell, 2013). Of these important diseases listed by the ANKC, all of the Great Dane breeders tested for hip dysplasia. However, even though hip dysplasia is a common problem in Labrador Retrievers and German Shepherds (Kimeli et al., 2015), only around half of breeders reported using Hip Scores on their breeding dogs. This may have been due to not all breeders completing this section of the survey, as registered breeders of Labrador Retrievers must have their breeding dogs hip scored, although this is not mandatory for German Shepherd breeders in Australia (Hedberg, 2016; National Labrador Retriever Breed Council, 2016). Further investigation into the reasons behind decisions to undertake genetic and health testing are required. Such knowledge is likely to help the efforts to increase the number of breeders undertaking tests on their breed stock.

6.4.4. Sire Selection

Only a small portion of the breeders surveyed used their own sires. When seeking sires, breeders preferred "distant" over "local" sires, and where possible, still preferred to meet and interact with the sire. Travelling distances to find sires might be required to ensure genetic diversity, which is particularly important for closed breeding lines. The location of the sire was a low priority for breeders, but conformation, size, pedigree, temperament, that the sire complements the dam, and the sire produces healthy puppies were all considered important in their decision. Only a small percentage chose to use artificial insemination methods. There are both advantages and disadvantages of artificial insemination (fresh or frozen semen) and natural mating in dogs. Advantages include an increased level of hygiene (Thomassen and Farstad, 2009) and safety, as well as long term storage and the high number of usage per sire (Vishwanath and Shannon, 2000). Natural mating in dogs may result in reduced whelping rates compared to artificial insemination using frozen semen (Gill et al., 1970). Disadvantages of artificial insemination exist and include a limited shelf life, expensive to store and disruption of sperm numbers

(Vishwanath and Shannon, 2000). Artificial insemination using frozen semen results in a smaller litter size compared to artificial selection using fresh semen (Linde-Forsberg and Forsberg, 1988; Linde-forsberg and Forsberg, 1992). Artificial insemination may lessen the likelihood that the dam would become sick or injured during impregnation, allowing a more successful pregnancy due to reduced stress. If not already, details surrounding artificial means should be discussed between breeders and veterinarians given the animal welfare implications for the bitch.

6.4.5. Limitations and Future Work

There were a number of limitations of the survey, particularly the low number of breeders who participated. Given the nature of the survey where participants self-selected, non-response bias cannot be calculated however, the results may be representing participants who were responsible and conscientious breeders registered with the ANKC. Thus, the findings reported here do not necessarily reflect the priorities or practices or all purebred breeders across Australia, nor represent all of the different breeds of dogs. It is equally important to understand and compare the practices of non-registered Australian dog breeders (Korbelik et al., 2011) as they are likely to differ in their breeding practices.

A number of important questions were unintentionally omitted from this study. For example, future studies should ask for the number of puppies born alive per year, whether it was more likely for the dam/(s) to give birth naturally or by caesarean, and other factors which may be important for specific breeds (i.e., hunting/retrieving capabilities for gundogs). Some issues were discovered with several questions that could also be improved for future studies. For example, our questions relating to priorities and practices were not breed specific, which did not allow breeders that bred more than one breed to be distinguished. In relation to accessing sires, there was some confusion as to the definition of *distant sire* given what people constitute as “distant”, and their willingness to travel (or import semen) are likely to vary among breeders and especially breeders of rare breeds. The survey also highlighted that some breeders wanted to undertake physical or DNA testing for their dogs but there were reasons as to why this was not conducted. For some, it was not physically possible (i.e., the breeder was in a remote location), tests were unavailable within Australia or there were no specific tests available. The expense of such testing may also influence decisions. We recommend that those involved with dog breeding and breed maintenance (i.e., veterinarians, ANKC) discuss options that are available to breeders so such tests can be accessed.

6.5 Conclusion

This study represents a step toward understanding breeding stock selection and breed priorities of Australian purebred dog breeders. Emphasis was given to breeding priorities and practices surrounding the dam, given the important role maternal care has on the development of puppies. These findings provide useful insight into dog breeding, and provide information that may be helpful for dog breeding groups and governing bodies (such as state governments, the ANKC) to manage breeding and breeder education. For example, the impact of caesarean births on mothering ability could be addressed to highlight the importance the mother has on puppy health and behaviour throughout the puppy's life. A significant association between ANKC breed group, the number of dogs the kennel bred, and whether the breed was brachycephalic on breed priority was found. This suggest that a "one size fits all" model for selecting and managing breeding stock is not appropriate. Importantly, it seemed that many breeders did not prioritise the maternal care behaviour of the dams. Emphasis of maternal care as a selection factor (for subsequent breeding of a bitch) should be made more prominent to breeders due to the impact it may have on puppy stress-related behaviour later in life.

Chapter 7. Discussion and Conclusion

7.1 Introduction

The aim of undertaking the studies described in this thesis was to better understand maternal behaviour in the domestic dog, and to determine the influence of maternal care on stress-related behaviour in puppies. If maternal care, like in other species, was found to influence stress responses in puppies, then selecting dams for maternal behaviour may be an important consideration for dog breeders, as this may help reduce the number of puppies relinquished due to stress-related behaviours.

In Chapter 3, maternal care in the domestic dog was reviewed, in particular dam presence, nursing, contact and anogenital licking, as described previously in a number of studies (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Wilsson, 1984; Grant, 1987; Malm and Jensen, 1997; Nagasawa et al., 2014; Guardini et al., 2015; Foyer et al., 2016). The review showed that maternal care behaviour has not been comprehensively described (i.e. short periods of observation to describe an infrequently occurring behaviour), and within litter differences are rarely accounted for. Dogs are born deaf, blind, and virtually immobile (Kendrick et al., 1997), therefore the neonatal period (first two postnatal weeks) is particularly important for maternal behaviour, yet this period of development has received little research attention. As described in Chapter 2, the impact of maternal care (namely licking and grooming) on HPA axis development is critical in the first 10 days postnatal in rats (Levine and Lewis, 1959; Meaney and Aitken, 1985; Myers et al., 1989; Liu et al., 1997; Caldji et al., 1998). As there are many similarities between rats and canines (e.g. young both rely heavily on their mother for survival, are usually born with littermates, similar developmental periods), comparison of the two species appears justified.

Evidence that dam maternal behaviour can influence puppy anxiety later in life has been recently reported (Tiira and Lohi, 2015; Foyer et al., 2016). However, inconsistencies in sampling methodology make it difficult to compare studies. This applies to dam behaviour (i.e. differing number of observation periods used and length of observation periods) and potential future offspring behaviour (testing age and testing apparatus differs). Maternal behaviour is important for consideration given that breeders are responsible for selecting breed stock. Therefore, the aims of this thesis were to: 1) comprehensively detail the distribution and frequency of maternal behaviours in domestic dogs, whilst taking into consideration inter- and intra-litter differences, 2) determine whether there are links between maternal behaviour and offspring stress-related behaviour in domestic dogs, and 3) understand reasons behind dam selection, in particular relating to maternal behaviour, by Australian purebred dog breeders.

7.2 Summary of Main Findings and Implications

7.2.1 Influence of methodology on understanding of maternal care

To date, a diverse and often simplified methodology has been employed in studies describing maternal care in domestic dogs. For instance, in studies observing interactions between dam and puppy, sampling periods have ranged from one to 14 hours per observation day (Rheingold, 1963; Grant, 1987; Korda and Brewinska, 1977a, 1977b). Differences in sampling period used are likely to limit the conclusions that can be reached. To determine which sampling method may be considered representative of a 24-hour period, a variety of sampling methodologies were compared to a 24-hour period for maternal care behaviour. Maternal behaviours observed included frequent behaviours (dam presence, nursing, contact) and infrequent behaviour (anogenital licking), described for both between and within a litter. A one hour period (comprised of four randomly selected 15-minute periods within a 24-hour period) was the optimal sampling period that best represented frequently occurring behaviours, while a longer sampling period (12-hour consecutive daylight period) was necessary to accurately describe anogenital licking.

It was determined that not all behaviours need to be observed for a 24-hour period to obtain an accurate representation of their occurrence. This, of course, depends on the behaviour of interest. This finding has important applied benefits, such as reducing observation time which reduces the total time researchers and breeders need to observe litters.

7.2.2 Differences in maternal care behaviour of dams

Differences in maternal care-giving behaviour can alter future offspring development and behaviour. With six different breeds and 10 domestic dog litters, differences were observed in duration of nursing, contact and anogenital licking between litters. However, there were no differences observed within a litter. This may suggest that the dam interacts equally with all puppies within the litter. This finding should be tested using a larger sample.

Studies presented in this thesis suggest that dams differed significantly in maternal care. This was found despite a small number of dams observed. In particular, dams differed in level of nursing, contact and anogenital licking. Differences in maternal behaviour have been previously documented (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a and 1977b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016). However, these studies used a variety of methodologies and behavioural definitions which makes maternal care behaviour difficult to compare across studies. Differences in maternal care behaviour (nursing, contact and anogenital licking) in this study were accurately represented as the sampling methodology was rigorous. Maternal care should be taken into consideration for breeders when selecting stock, in addition to the more obvious factors of overall temperament and breed type. Previous differences in behaviours in different dog breeds have been documented, such as play fighting (playful biting and pawing) in response to handling (Scott and Fuller, 1965). Cocker Spaniels showed consistently less play fighting behaviour over the first postnatal year than Wire Haired Terriers, Beagles, Shelties and Basenjis (Scott and Fuller, 1965). As only six breeds were represented in this thesis, further observations are needed to determine whether findings relating to maternal behaviour extend to other breeds.

7.2.3 Influence of maternal care on stress-responses in puppies

The isolation test, an established test where puppies are separated from the dam and litter, was used to elicit a stress response in puppies. The difference in anogenital licking duration on postnatal day 6 was compared to puppy behaviour and heart rate during the test. Significant differences in activity, vocalisation and heart rate were apparent between puppies. Of note, an increase in anogenital licking on day 6 was associated with decreased activity and vocalisation latency, but a smaller difference in heart rate (post-test heart rate minus pre-test heart rate) as a two-way interaction with parity. These results are consistent with puppies licked more by their mothers exhibiting a stress response (e.g. longer latency to vocalise) more quickly, but then recovering more quickly as the heart rate increase post-test was lower in these puppies. Further research is necessary to determine whether the onset of fear-related avoidance behaviour occurs at different ages in puppies, and whether the recovery period of puppies is shorter in puppies licked more often within the first 10 postnatal days.

Predicting a dog's response to stress later in life based on puppy behaviour has been explored previously (e.g. Beaudet et al., 1994; Wilsson and Sundgren, 1998b; Seksel et al., 1999; Slabbert and Odendaal, 1999; Gazzano et al., 2008; Asher et al., 2013; Kutsumi et al, 2013; Riemer et al., 2014; Rocznik et al., 2015; Robinson et al., 2016). However, the success of predicting future stress response remains low, with few studies addressing the interaction between puppy and dam and its effect on future behaviour, and/or dogs tested were conducted when the puppy was over one year of age. Puppy behaviour may be determined in the first few postnatal weeks, as reported in rat literature. There is a large investment from dog breeders to match a puppy with the new owner, or develop puppy behaviour for specific roles (i.e. guide dogs). If the behaviour of puppies is determined during the first few weeks, there is a potential that there will be a reduced amount of time and cost invested by the breeder for that dog as breeders can predict, for example, which puppies are more likely to respond better in guide dog testing. The early environment, in particular interaction between dam and puppy, may impact future puppy behaviour, as highlighted in Chapter 5. The ability to observe simple behaviours of seven-week old puppies (i.e. exploration) to predict adult behaviour may allow breeders to determine puppy behaviour more easily. Further research is needed to determine whether the stress response is normal (i.e. a stress response is elicited but the recovery rate is short) for dogs when they are older. Behaviour of young puppies (7-8 weeks old) may allow the breeder to better match puppy with an owner. Puppies with pronounced stress behaviour may be better suited to owners which can spend more time with the animal. This might ultimately help match the puppy to its owner and give pre-warning to the owners about the behaviour and temperament of the puppy, reducing the chance that the owner will relinquish the dog.

7.2.4 Importance of maternal care when choosing dam stock for breeding

The breeder survey (Chapter 6) was the first step in understanding breed priorities of Australian purebred dog breeders. This study highlighted that maternal care was not always considered a high priority when selecting dams, and hence other factors may have more importance placed on them than maternal care. Predictors, such as the Australian National Kennel Club (ANKC) breed group, size of the kennel (i.e. the number of breeds) and whether the breed was considered as brachycephalic or not, were important in altering breed priority relating to Maternal Care, Offspring Potential, Dam Temperament, and Dam Genetics and Health. However, the ANKC breed group was the only variable to alter the importance placed on maternal care. Maternal care scored more highly in Toy and Hound breeding groups than Terriers, Gundogs, Working dogs and Utility dogs. Fifty percent of brachycephalic dogs in the study were classed as Toy breeds. It is likely that brachycephalic breeding dams need more help during and after parturition, which might explain why breeders consider maternal care to be more important.

A change of breed purpose has occurred from working to companion dogs and it may also be likely that breeders need to be made aware of maternal care as a breed priority and include it when considering breed stock. Maternal care is an important factor because it is likely to influence later offspring behaviour and this information should be provided to all dog breeders. This message could be incorporated in easily accessible web pages (i.e. on the ANKC website) or through breed clubs.

If maternal care is taken into consideration when choosing breed stock, it may be likely to reduce the number of puppies pre-disposed to reacting stressfully in certain situations. Dog owners may also benefit from an increased emphasis on maternal care. Dogs which do not display signs of anxiety such as circling, restlessness (Sherman and Mills, 2008) or increased barking (Notari and Mills, 2011), are less likely to be relinquished (Miller et al., 1996; Patronek et al., 1996; Salman et al., 2000; Segurson et al., 2005). This is positive for dog welfare and the human-dog bond. The pressure on animal shelters will also be alleviated following a reduced number of relinquished dogs, hence potentially saving money and space for other animals.

7.3 Problems Encountered and Limitations

Data were successfully collected and analysed in this thesis, however there were limitations which altered data quality due to sample representativeness. An ideal study would observe dams providing maternal care to puppies in a laboratory setting, where dams are of the same age, breed and health status are attended to by the same researcher, and where all dams are either first time mothers or produced the same number of litters previously. Additionally, an ideal study would have a large enough sample size to make a comparison between natural births and puppies born by caesarean. The next ideal study situation is to observe puppies born in a home setting under natural conditions (i.e. dam makes nest outside, puppies born outside and birth is not assisted by a human handler, with no interaction from breeder/human handler to nurture puppies for survival). However, neither of these ideal scenarios were possible and the problems encountered and limitations which arose during the study are now described.

7.3.1 Subject availability

Recruiting dog breeders to take part in the study proved challenging, despite numerous dog breeders being located in South Australia, Australia. A number of breeders were willing to have video equipment set up in their homes and have puppies tested at seven weeks of age, however the dam either did not come into season or did not become pregnant. In addition, breeders who had volunteered to take part in this study were not able to have the equipment in their house before whelping or contacted me after or on postnatal day 3.

The design of the study initially focussed on one breed of dog. However, due to the recruitment challenges described above it was not possible to observe the same breed of dog. Also, due to small sample size, it was not possible to control other variables such as age of dam. With a larger sample size, it would have also been interesting to divide puppies into caesarean or natural birth. Although significant differences were found between dams in this study, if the variables above were controlled, stronger evidence may be provided for the link between maternal care behaviour and stress behaviour in puppies.

7.3.2 Filming of puppies

Filming of litters also proved problematic at times. Set up of the camera and recording box required multiple cables connecting different equipment. Some dogs pulled cables from the recording box and some footage was missing before the dog breeder realised that a problem had occurred with the equipment. It was preferable that equipment was installed in the breeder's property before the dam had whelped, as this reduced dam stress when with her puppies. When the researcher left the breeder's property, the camera was positioned at the best angle for observation. However, when the dam whelped and the breeder intervened near the whelping box, there were times that the camera was accidentally moved and the position of the whelping box was changed which altered and reduced view of the dam and puppies. This made it difficult to observe all areas of the whelping box, and sometimes the puppies, during the first two postnatal weeks.

One aim of this thesis was to determine whether there were differences in maternal care within a litter. To achieve this, each puppy needed to be identified during the filming period. However, puppy identification was not always possible. Breeders were not deterred from applying their usual practices with puppies and were asked to identify puppies in a way they preferred (e.g. ribbon around the puppy's neck, a dot of liquid paper on different segments of the body). It was often difficult to determine an individual puppy because the dam was covering the puppy, the puppy was covered by other puppies or the puppy was lying in a ventral position. Some ribbons would fall off multiple puppies during the observation period and it was not until the breeder went back with the puppies that they noticed they were missing. As puppies grew older, the accuracy of puppy identification increased. There were additional issues relating to individual studies undertaken in this thesis, discussed below.

While litters were recorded, breeders were instructed to continue their usual interaction and duties with the litter. The breeder would remove the puppies from the whelping box and the puppies moved outside the view of the camera, and the puppy was recorded as out of sight. The dam would sometimes jump out of the whelping box with the puppy, and may have interacted with the puppy outside of view. Therefore, it is possible that some puppies had an additional amount of care from either dam or breeder (i.e. breeder holding the puppy) not recorded in the study. Although unlikely, this could have altered the results of maternal care and ultimately impacted outcomes of the isolation test.

7.3.3 Maternal care behaviour and stress related behaviour in puppies

Puppy stress behaviour was assessed once during the study. To be more confident with the results, an additional test to determine stress behaviour is necessary. The isolation test could be applied again when the puppies are older (approximately 12-16 weeks old). However, use of this specific test to determine stress behaviour may cause puppies to be accustomed to the novelty of the box, reducing accuracy of results. In addition, more variables are present when puppies are older. For example, after 7-weeks of age puppies are relocated to a new home with their new owner, presenting new variables which may also affect puppy behaviour. Another test may be required to assess behaviour in older puppies.

7.4 Future Studies

A total of 47,000 puppies and dogs were relinquished to the RSPCA across Australia between 1st July, 2014 and 30th June, 2015 (RSPCA, 2015). Of animals relinquished, 4,700 (or 10%) of dogs were euthanised due to behavioural problems (RSPCA, 2015).

To reduce the number of dogs and puppies relinquished to shelters, puppies responding with high stress and anxiety levels should be identified as early in life as possible to allow early interventions to reduce fear and anxiety. A common cause of dog relinquishment is stress-related behavioural problems (Miller et al., 1996; Patronek et al., 1996; Salman et al., 2000; Segurson et al., 2005). Although the impact of maternal care alone will not stop all anxious behaviours in puppies and dogs, the impact of maternal care on puppy behaviour is one step in reducing the number of puppies predisposed to stress.

7.4.1 Handler effect

The breeder or puppy handler is an important environmental effect for puppy development. This is because the breeder may have a large influence on puppy care and maintenance. It is common for the breeder to provide additional sustenance and assistance to the puppy for survival, including food, cleaning to stimulate urination and defecation and warmth. The amount of involvement the breeder has with the litter may alter dam maternal care behaviour and puppy development. Breeder interaction with puppies, removal from the litter environment, introduction of foreign animals, people and objects and handling the puppies often, can alter puppy response to stress later in life. Breeder interaction with puppies may have affected the way puppies responded in the isolation test. Puppies handled and stimulated (tactile stimulation, head held erect, head pointed down, supine position and thermal stimulation) explored more and were more active than their non-stimulated littermates during competitive situations (Fox, 1972). Puppies raised in a breeding kennel had a longer latency to vocalise, vocalised for a shorter period of time and spent less time exploring the environment in isolation compared to family raised puppies (Gazzano et al., 2008). Without a repeat measure on the breeder for their interaction with the dog, it is impossible to determine breeder effect in the current study. Future research should determine the interaction between breeder and litter in conjunction with observing the interaction between puppy and dam. The length of breeder interaction (time per day), quality of breeder interaction (e.g. is the breeder picking up and holding the puppies or just petting the puppy while it is lying in the box) and environment to which puppies are exposed may alter puppy behaviour later in life. The level of intervention and effect of these interventions on puppy development are not fully understood.

7.4.2 Determining the impact of maternal care on the development of stress-related behaviour and recovery from stress

Puppies licked more on postnatal day 6 had a shorter latency to vocalise and had a smaller heart rate difference in the isolation test, which was not expected. Response to an acute stressor may provide a survival advantage (Kirby et al., 2013). Puppies responding with a normal stress response in the isolation test may have enhanced neural function and memory performance, as observed in laboratory rats (Lupien and McEwen, 1997; Conrad et al., 1999). The developmental onset of fear-related behaviour may occur earlier in puppies licked more during the first 10 postnatal days. Fearfulness occurs at different times dependent on dog breed (Scott and Fuller, 1965; Morrow et al., 2015) which is possibly related to morphology of facial features (Trut et al., 2009). To determine the impact of maternal behaviour on developmental onset of fear-related behaviour, it would be necessary to repeat the study presented in this thesis with a larger, more controlled sample (i.e. same dog breed) and test puppies for a longer period of time.

Recovery from a stressful response may be shorter in dogs licked more during the first 10 postnatal days, as the difference in heart rate from pre- to post-test was smaller. The licking duration of mothers can reduce the length of time offspring is anxious. This may be due to differences in receptor density and receptor number involved in anxious behaviour, as reported in laboratory rats (Francis et al., 2002) where a smaller plasma corticosterone difference was observed with autoradiography occurred in puppies which spent more time with their mother. Frequent monitoring of physiological measures (i.e. cortisol, heart rate) during stress tests in dogs may assist in determining the pattern of recovery in puppies. It would also be useful to repeat the studies in this thesis using the same breed of dog to increase consistency of results.

7.4.3 Determining the impact of siblings on future puppy behaviour

In this thesis, the impact of sibling interaction on puppy development, and in particular, the development of stress responses, were not assessed. Puppy interaction with siblings can influence individual physical, psychological and behavioural development (Hudson et al., 2011). In particular, playfulness, chase-proneness, cupidity/fearfulness and aggression in German Shepherd dogs was largely influenced by littermates (Strandberg et al., 2005). Again in German Shepherd dogs, puppies with fewer siblings tended to be have higher confidence scores. Littermates are also important in developing adequate agonistic behaviours, such as aggressive play and sparring, while puppies without littermates are unlikely to learn these skills (Lindsay, 2001). Further research is needed to determine the impact that littermates have on puppy behaviour later in life. It is likely that littermate interactions also ultimately affect puppy personality, which would alter the behaviour observed within the isolation box. Littermate behaviour may give more information regarding puppy behaviour during isolation testing; for example puppies exploring may indicate a more active animal rather than a non-stressed animal. Future research should focus on individual differences in puppy behaviour to determine stress response of individual puppies.

7.4.4 Determining the representativeness of the isolation test with seven-week old puppies

Stress responses in domestic dog puppies may differ depending on the test used, behaviour observed or age at which puppies are observed. Short stressful tasks (e.g.

exposure to a new environment or a new person), with 17 seven-week old puppies and 10 adult dams resulted in an increase in salivary cortisol post-test compared to a control sample (Svobodova et al., 2014). Adults had a lower concentration of cortisol compared to the puppies. The animals also had their IgA levels assessed to determine stress (Svobodova et at., 2014). IgA concentration decreases when an individual is stressed. When salivary IgA was recorded pre-and post-test, puppies had a larger difference in IgA concentration compared to adults, suggesting that puppies had a higher response to stress than adults (Svobodova et at., 2014). In contrast, Palazzolo and Quadri (1987) did not observe any changes in cortisol or thyroxine due to low ambient temperature in eight-week old puppies. Exploration behaviour, as a measure of emotional state (LeDoux, 1986), was not prominent in puppies until approximately 12 weeks of age, but fear of human handlers was observed at five weeks of age (Scott and Fuller, 1965). A difference in salivary cortisol was observed in puppies differing in onset of fear-related avoidance behaviour (Morrow et al., 2015). Although three breeds were tested, Cavalier King Charles Spaniels portraying fear-related avoidance behaviour had a greater cortisol response compared with puppies which did not elicit the behaviour. Cortisol concentration decreased in all breeds from postnatal week four to 10 (Morrow et al., 2015), hence differences in cortisol concentration between studies may be a reflection of the time of testing. There is contrasting evidence as to when stress responses in puppies are observed, and further work is needed to determine when stress responses are elicited.

The relationship between testing dogs as puppies and adults has provided contrasting outcomes. Adult fearfulness measured using 17 scores assessing five factors (distraction, general performance, sensitivity, fearfulness and fearfulness accompanied by high activity) could be predicted by testing three month old puppies (Goddard and Beilharz, 1983), however as the puppy aged from first testing onwards the correlation became higher. Wilsson and Sundgren (1998b) found that puppy behaviour at eight weeks of age did not predict successfulness of suitability of service dogs tested at 15-20 months of age. Fear-related behaviour appears at different times (Goddard and Beilharz, 1983; Morrow et al., 2015), therefore determining an age at which to test the puppies needs to be determined, although it does appear that puppies need to be tested when older. It is important to test puppies at different age intervals to observe whether a correlation between ages exists.

7.5 Conclusion

This thesis observed a small portion of possible reasons contributing to undesirable behavioural problems in dogs. Maternal care differs between dams and results in differences in puppy response to stress later in life. Maternal care is recently becoming an area of interest which may impact stress behaviour in dogs and therefore play a role in predisposing puppies to anxiety. The importance of maternal care may be a factor not always considered when choosing breed stock, thus breeders and potential owners should be educated on the importance of maternal care behaviour. Commitment to understanding the importance of maternal care and its impact on future dog behaviour may be important in reducing anxiety-related behaviours in dogs, ultimately improving the human-dog bond and potentially reducing dog relinquishments. Further research is needed to determine what constitutes a ‘good’ mother, which maternal care behaviours can be used to determine a ‘good’ mother, and whether other behaviours can be used to predict a ‘good’ mother rather than observing litters over a period of time.

Appendix 1: Breeder Survey questions

Breeder Choices Survey
(Please circle your answers)
About you

1. Gender: Male Female

2. Age: ________

3. Postcode: ________

4. Highest level of education:
   High School TAFE Undergraduate Post-graduate
   Other? ______________

5. What breed (/s) of dog (/s) do you breed?
   ____
   ____
   ____

6. How long have you owned your breed/s?
   ____
   ____
   ____

7. How long have you bred dogs for?
   _________ years

8. How many litters of puppies do you normally breed per year?
   _________litters

9. Do you show dogs?
   Never Seldom Often

10. Do you participate in other ANKC recognised competitive dog sports?
    Never Seldom Often

11. Are you a member of your state or territory canine council, which is a member of body of the Australian National Kennel Council (ANKC)?
    Yes No

12. Are you a member of an ANKC recognised working dog association?
    Yes No

13. Are you a member of a breed club, or a group if no breed club exists?
    Yes No
14. How many bitches do you currently own that you breed from or hope to breed from in the future?

    ____

15. How many dogs (male and female) in total do you currently own?
    _______dogs &______bitches

16. Where do you advertise the puppies when they are ready to be sold? *Please tick as many as applicable.*
    ☐Gumtree ☐Trading Post ☐DogzOnline
    ☐Other website (please list) ______________ ☐ Word of mouth
    ☐Newspaper ☐Other (please list)________________________

About the dam (/s)

17. On average, how often will you breed from each bitch?
    _________ times in their life

18. At what age do you usually first breed from your bitches?
    ____

19. At what age do you usually last breed from your bitches?
    ____

20. Do some of your bitches usually live with somebody else?
    Yes No

21. Where do your bitches usually live?
    Outdoors Indoors Both

22. How important are the following in your decision to breed from an ideal dam?
Please tick once for each statement

	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
My ideal dam has outstanding conformation according to the breed standard					
My ideal dam is within the accepted size according to the breed standard					
My ideal dam has an optimal temperament for the breed					
My ideal dam has passed all required and recommended health tests for her breed					
My ideal dam comes from a line of healthy, long lived relatives					
My ideal dam is friendly to dogs, other animals and people					
My ideal dam is excitable					
My ideal dam is obedient					
My ideal dam is confident					
My ideal dam has a strong bond to humans					
My ideal dam is trainable					
My ideal dam has an outstanding pedigree					
My ideal dam has naturally conceived with ease					
My ideal dam has whelped with ease					
My ideal dam has an excellent maternal instinct towards her puppies					
My ideal dam produces sufficient milk to raise her puppies					

23. Are tests available for genetic disorders within your breed/s?
Yes No

24. Do you undertake DNA testing of your breeding animals for any genetic disorders? If yes, which ones?

25. Do you undertake physical testing for genetic disorders, such as heart and eye examinations? If yes, which ones?

26. How important are the following in your decision to remove a dam from your breeding stock?
Please tick once for each statement

	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
I would not breed from a dam if conception and whelping were difficult					
I would not breed from a dam if her maternal behaviour was not ideal					
I would not breed from a dam if her puppies did not conform closely to the breed standard					
I would not breed from a dam if the temperament of her puppies was not ideal					
I would not breed from a dam if she had rejected her puppies					
I would not breed from a dam if some of her puppies had a significant genetic fault					
I would not breed from a dam if she was aggressive towards strangers					

About the sire

27. Where are you most likely to access a sire?
Own sire Local sire owned by someone else
Distant sire owned by someone else

28. If the sire is sourced from elsewhere (not own sire), do you personally spend time with the sire observing his temperament and structure before mating him to your bitch (/es)?
Yes No Not possible

29. Rate the following factors for their importance in choosing a sire to breed to your dam?
Please tick once for each factor

	Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
My ideal sire lives conveniently close to me					
My ideal sire has outstanding conformation according to the breed standard					
My ideal sire is the accepted size according to the breed standard					
My ideal sire has an outstanding pedigree (desired ancestors in common with the dam)					
My ideal sire has an optimal temperament for the breed					
My ideal sire complements my bitch's virtues and does not share her faults					
My ideal sire has produced puppies which are healthy and conform closely to the breed standards when mated to other bitches					

30. How important is the coefficient of inbreeding (COI) of the litter when determining which bitch and dog will be mated? *Please circle one*
Very important   Slightly important   Neutral   Slightly unimportant
Not important   I do not know what COI is

Other information

31. Is there any further information you would like to share regarding your breeding animals or breeding practices?

Thank you for completing this survey!

Appendix 2: Published Journal Article 1

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Canine Research

The influence of maternal care on stress-related behaviors in domestic dogs: What can we learn from the rodent literature?

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ABSTRACT

An estimated 40% of dogs living as companion dogs are believed to exhibit some form of anxiety or stress-related behavior. Although this represents a significant welfare issue, our understanding of the origins of anxiety in dogs remains limited. Genetics, environment, and training methods have all been investigated, yet little attention has been paid to the care provided by the mother. Research conducted with altricial species, that rely heavily on maternal care for survival, suggests that early maternal care behaviors play an important role in the development of the infant and thus, behavior and temperament later in life. The most critical maternal behaviors include contact, nursing, licking (particularly anogenital licking which stimulates urination and defecation), punishment, thermoregulation, and movement. In domestic dogs, rapid neurological development occurs between postnatal days 3 and 16, yet investigations fail to measure or acknowledge the role that maternal care has during this critical window, or how the experience of puppies during this time influences behavior later in life, including response to stressful events. Evidence from the rodent literature indicates profound effects of maternal care on neurological and behavioral development. Although there may be differences in maternal behavior between rats and dogs, the underlying physiological mechanisms underpinning the programming of stress-related behavior are likely to be similar. For instance, the hypothalamic-pituitary-adrenal axis or stress responsiveness pathways are profoundly altered by maternal behaviors, and these changes are conserved throughout adult life. In this review, we examine the literature related to maternal care in canids alongside the literature related to maternal care in rodents and provide evidence that maternal care is critical to the healthy development of domestic dogs. Emphasis is placed on methodologies for quantifying maternal care and on the physiological mechanisms that might underpin behavioral changes induced by different amounts and types of maternal care.

Introduction

Common undesirable behavior traits in dogs relate to anxiety, including separation-related behavior, generalized anxiety, and noise phobia (Overall et al., 2001; Overall and Dunham, 2002; Marston et al., 2004; Bennett and Rholf, 2007; Buckland et al., 2014; Serpell and Duffy, 2014). Anxiety related behaviors affect approximately 20%-40% of domestic dogs (Simpson, 2000). Such behaviors are not only displeasing from the owner's perspective but also represent a major welfare problem for the dog. Indeed, separation-related behavior and noise phobia have been rated in the top three most important companion dog welfare issues (Buckland et al., 2014). There are many factors that can contribute to undesirable behaviors in dogs including genetics [e.g., breed characteristics and inheritance (Scott and Fuller, 1965; Goddard and Beilharz, 1983; Saetre et al 2006)], the environment [e.g., prenatal environment, parental behavior, husbandry methods, interaction with conspecifics, humans, and new experiences (Hetts et al., 1992; Jagoe and Serpell, 1996; Kobelt et al., 2007)], as well as exposure to training and training methods (e.g., Jagoe and Serpell; 1996; Hiby et al., 2004; Rooney and Cowan, 2011). Many of these factors have been explored in an attempt to better predict desired traits later in life for individuals kept for breeding, exhibition, work or recreation

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animals, or household pets (Slabbert and Odendall, 1999, Hennessy et al., 2001, Svartberg, 2002, Foyer et al., 2013).

The success of accurately predicting stress behavior in adult dogs remains low (Beaudet et al., 1994; Wilsson and Sundgren, 1998; Seksel et al., 1999; Slabbert and Odendaal, 1999; Asher et al., 2013; Kutsumi et al., 2013; Riemer et al., 2014). Most studies focus on juveniles, with few addressing what affects may arise as a result of experience during early development, particularly the interactions between the dam and the puppy. In altricial species, such as the dog, offspring are unable to care for themselves at birth, are usually born deaf and blind, and have limited movement (Kendrick et al., 1997). Thus, the effect of maternal care and the early postnatal environment may have marked effects on subsequent stress-related behaviors. The correlation between maternal care in puppyhood and anxiety development in dogs has been identified by Tiira and Lohi (2015). In that study of over 3,000 dog owners, dogs that had an owner-reported lowered maternal care score were significantly ($P < 0.0001$) more likely to be at risk of anxiety (Tiira and Lohi, 2015). Direct evidence for a link between maternal behavior and puppy anxiety later in life has been recently documented (Foyer et al., 2016). Maternal behavior of 22 German shepherd litters was observed and coded every second hour (for one hour), once a week for the first three postnatal weeks. The dams differed in the level of maternal care and were separated for analysis into a low or high level of care. At 18 months of age, the behavior of the puppies was significantly affected by the level of maternal care given. These studies (Tiira and Lohi, 2015, Foyer et al., 2016) are the first to highlight a link between maternal care in puppies and later anxiety in dogs. Unfortunately, maternal care in domestic dogs, in particular within the critical first two weeks postnatally, remains poorly understood and largely ignored by the scientific community. Maternal care as a predisposing factor for dog anxiety is a relatively novel concept in dogs but has been well described in other altricial species.

In this article, we provide a review of the literature relating to maternal care as an early postnatal environment in domestic dogs. As our understanding of the developmental period and maternal care in dogs is limited, we draw parallels with rodents. Although we acknowledge that maternal behavior may differ between species, rodents are a useful model because, like dogs, offspring are heavily reliant on the dam; the physiological mechanisms underpinning stress-related behavior are likely to be similar; and they have been well studied (because of their short lifespan). We also discuss the methodology that has been used in studies describing maternal care in dogs and suggest ways in which this could be improved. To avoid confusion, we refer to "puppy" as being the offspring of a domestic dog, whereas "pup" refers to offspring of the rodent, the domestic rat.

Maternal care

Parents not only provide the genetic material for their offspring but also play a fundamental role in the offspring's environment. Thus, they are arguably a major contributor to the development of their young, including undesirable behaviors. For the young of altricial species, the early social and physical environment (usually in a burrow or nest) is determined largely by interactions with the mother (Francis and Meaney, 1999). Even subtle variations in maternal care, for example licking, can have a profound effect on development in rodents, and also most likely in dogs.

Maternal care in dogs

During a natural birth, the dam will lick the placenta off the newly born (neonate) puppies, eating the placenta remains, and biting the umbilical cord of the puppy (Jack and Watson, 2008). Puppies are likely to nurse while the dam is still whelping other puppies in the litter. From birth until the puppies are approximately four weeks old, the dam is needed to stimulate urination and defecation (through anogenital licking), feed the puppies, and provide a heat source to allow the puppies to maintain a stable body temperature (Walker, 2010). The neonatal period, also known as the primary period, begins at birth until postnatal day (PND) 12 (Scott and Fuller, 1965), or PNDs 3-16 (Battaglia, 2009). This period is important as puppies are sensitive to thermal and tactile stimulation, motion, and locomotion (Fox, 1972; Dunbar, 1979; Hoffman et al., 2004), and therefore, detrimental stimulus to the puppy within this period can alter the animal's behavior later in life. Between PNDs 3-16, there are rapid neurologic growth and development occurring in puppies (Battaglia, 2009). Although the puppies are relatively young and immature, the dam will protect them from animals and unknown humans posing a threat. The following behaviors have been documented in the early litter environment: contact, nursing, licking, play, movement to and away from puppies, and punishing behaviors (Rheingold, 1963). Punishing behaviors of the young are classified as when the dam growls or barks at the puppy, snaps or bares her teeth towards them, picks up the puppy with her mouth and shakes it, holding down the puppy with her paw or pouncing on the puppy in a nonplayful manner (Rheingold, 1963). Regurgitation of food by the bitch to puppies also occurs and is most likely brought on by the begging signal portrayed by the puppy, although regurgitation is not common (Lord et al., 2013).

By the end of the fourth week of the puppy's life, they have coordinated walking, autonomic thermoregulation, visual depth perception, and begin the initial stages of voluntary consumption of moistened solid food (Lawler, 2008). During the fifth postnatal week, the dam starts to frequently walk away from the nest, the whelping area, thereby signaling weaning has commenced (Wilsson, 1984). The socialization period begins preweaning when the puppies are three weeks of age and lasts until they are approximately 12-14 weeks of old (Battaglia, 2009) with a "critical period" (a time when the smallest amount of negative stimulus may have a large effect on later life behavior) occurring between the ages of 1-6 weeks (Scott and Fuller, 1965). However, there is conflicting thought about the specific time period at which socialization ends. Differences in fear-related avoidance behavior varied between German shepherds (39.4 ± 6.5 days), Cavalier King Charles spaniels (55.1 ± 3.1 days), and Yorkshire terriers (42.2 ± 2.5 days) (Morrow et al., 2015). During the socialization period, the interaction of the puppy with novel objects, animals, and individuals allows familiarity with those components later in the puppy's life (Scott et al., 1974).

Compared to wild canine species, domestic dogs are considered to have a reduced inclination for maternal behavior (Lord et al., 2013), and therefore, human intervention may be required for puppy survival. Although dam-puppy interactions have been documented (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Wilsson, 1984; Lund and Vestergaard, 1998; and Walker, 2010), there is yet to be accurate description and documentation of these behaviors within the neonatal period.

Methodology used to study maternal behavior in dogs

There have been few reports of maternal care in domestic dogs. Of those that exist, many have used differing and potentially limited methodologies for assessing behaviors, leaving many questions unanswered. Altmann (1974) recognized the need to be rigorous in applying appropriate methodologies. For example, distinguishing between an "event" (an instantaneous behavior such as an animal lies down) or a "state" (a behavior with an appreciable duration such as an animal is lying down) can affect the frequency of the behavior observed (Altmann, 1974). The onset and termination of the session is also important because the length of the session affects the behaviors observed, and observer fatigue can alter results recorded. Altmann's (1974) comments related to direct observation, as opposed to video cameras that are now widely used in observational studies and should lessen these concerns. Using video footage may enhance the number and accuracy of observations, as well as decrease observer fatigue. It allows the observer to correctly describe behaviors which may be occurring simultaneously. Video also allows measurement of interobserver reliability. The length and type of behavior recorded (event vs. state) rely on the hypothesis of the study and what questions are to be answered.

Methods for observational sampling have been described by Altmann (1974), and sampling methods relevant to maternal behavior are briefly described here. Maternal care behaviors, such as anogenital licking, occur infrequently, and therefore, the use of state sampling increases the chances of accurately describing the duration and frequency of the behavior, rather than only describing the frequency which may be misleading. The focal-animal method of sampling is used to record all occurrences of a specific action of one individual or a group of individuals. The length of each observation period is recorded, and the amount of time the individual/group is within view is also recorded (Altmann, 1974). This method is recommended to determine the percent of time, rates and durations, chronological constraints, and neighbor relationships for a particular behavior (Altmann, 1974). Instantaneous and scan sampling is recommended to determine the percent of time a particular behavior is occurring (Altmann, 1974). This method requires an observer to record the behavior of an individual during preselected sessions and is useful in calculating the percent of time the behavior is occurring within the sample period. Specifically, the method used in an ethological study should accurately describe those behaviors occurring less frequently, and therefore, the sampling time is of high importance.

There have been different methods used to describe dam and puppy interactions within a litter of dogs in the early postnatal environment (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a, 1977b; Wilsson, 1984; Grant, 1987; Malm and Jensen, 1997; Nagasawa et al., 2014; Guardini et al., 2015). For example, Rheingold (1963) recorded the maternal behavior from PND one where observers recorded the interactions between dam and litter using ad libitum and focal-animal sampling methods. *Ad libitum* sampling was also used by Scott and Fuller (1965) and Wilsson (1984). Behaviors recorded are listed in Table 1. In particular, licking of the puppies occurred between approximately 0% and 1.2% on PND one; however, these numbers are estimates extracted by the authors of this article (quantitative data were not presented). It is likely that there are biases in results if the duration of recording is not long enough to observe less frequent behaviors (Rheingold, 1963; Scott and Fuller, 1965; Wilsson, 1984; Guardini et al., 2015). For example, recording the dog litters for a short period may not accurately describe the number of times the dam licks the puppies. If the sampling period is taken at random, it is difficult to compare studies because if behaviors are occurring at particular times of the day, these different sampling periods may represent differences in the behavior (Altmann, 1974).

Sampling using the focal-animal technique requires the length of the sessions to be sufficiently long to provide adequate estimates of nonfrequent behaviors. It is likely that anogenital licking, which is not a frequent behavior, will not be represented accurately in the studies described previously, where the litters were observed for less than an hour a day. Korda and Brewinska (1977a, 1977b) have also investigated maternal behavior in the dog (see Table 1). The sampling method was instantaneous and scan sampling, where maternal behavior within the litter was recorded as states during a preselected session. The method creates a greater margin of error for duration estimates as opposed to other methods; however, the percent of time within the sample period is accurately described. It is likely that the results presented by Malm and Jensen (1997), Nagasawa et al. (2014), and Foyer et al. (2016) are also more precise given the observation period was longer.

Management practices and puppy development

Breeding management practices may alter the dam's behavior toward her litter. A common practice for dog breeders is to remove a puppy from their littermates and the dam and hold the puppy for a short period (Battaglia, 2009). In a study (Gazzano et al., 2008) to assess the effect of handling on later behaviors, 43 dogs were separated into four groups: nonhandled puppies raised in a family, handled puppies raised in a family, nonhandled puppies raised in a professional breeding kennel, and handled puppies raised in a professional breeding kennel. Handled puppies were removed from the litter between PNDs three and 21, massaged and turned on their back where an abdominal massage was undertaken before the puppy was placed back into the litter. The outcome of an isolation test indicated that handled puppies seemed calmer, showed a longer latency to vocalize, and spent significantly more time in exploratory activity compared to nonhandled puppies (Gazzano et al., 2008). Using the Bio Sensor stimulation exercises (tactile stimulation, head held erect, head pointed down, supine position, and thermal stimulation) once a day on each puppy, it was highlighted that handled puppies showed more tolerance to stress than puppies not handled (Battaglia, 2009).

Maternal care in rodents

Rats are a particularly good model to determine possible links between maternal care and effects of stress for a number of reasons. The short lifespan (Holliday, 1989) and development of the rat allows for lengthy, descriptive observations of their development. Ultimately, the large amount of research into physiological changes in the brain, due to altered maternal care, has been extensively studied in the rat (Liu et al., 1997; Caldji et al., 1998; Caldji et al., 2000). The mother retrieving pups which have moved away from her and the lactating posture are important maternal behaviors to allow a more successful attachment of the pups to the nipples, and ingestion of milk. The ideal posture is arched-back nursing, where the mother's hind feet are spread and the back is raised in an arch (Fleming and Rosenblatt, 1974). Other maternal behaviors include licking and grooming, nursing postures, retrieval of pups as well as the retrieval latency (how long the mother will take to retrieve the pups which has moved away from the litter), nest building (Fleming and Rosenblatt, 1974), carrying the pups and blanket nursing, where the mother lies over the offspring with a low dorsal arch posture (Pryce et al., 2001). In a laboratory setting, rat pups' ears open around PND nine, eyes open at PND 14, and are weaned at about PND 21 (Inagaki et al., 2013; Mucellini et al., 2014; Williams et al., 2014). Low licking/grooming and arched-back nursing (LG-ABN) mothers are considered as those which are at least 1 standard deviation less than the mean, whereas high LG-ABN mothers are classified as those mothers which are at least 1 standard deviation more than the mean for both licking/grooming and arched-back nursing (Liu et al., 2000). These significant differences in maternal behaviors were found in the first eight days of offspring life, although the overall time the mother was in contact with the pups did not differ. Interestingly, differences in licking and grooming have been found within the first-week postpartum with peak occurrences observed within the first few PNDs (Champagne et al.,

Table 1
Maternal care methodology in domestic dogs comparing sampling method, sampling duration, behaviors observed, and description of data

Study	Sampling method	Sampling period (postnatal days)	Sampling duration	Behaviors observed
Rheingold (1963)	Ad libitum Focal-animal sampling	1-68	15 minutes periods × 4 times a day between 0800 and 1800 hours	1. Contact between dam and litter 2. Number of puppies in contact with the dam 3. Nursing duration 4. Number of puppies suckling 5. Licking of the puppies by the dam 6. Number of times the dam went to the puppies 7. Number of times the dam went away from puppies 8. Number of times puppies went to dam 9. Number of times the dam punished the puppies 10. Time the dam spent in the whelping box
Scott and Fuller, 1965	Ad Lib	1-49	10-minute observations, weekly	1. Nursing behavior 2. Retrieving test 3. Weaning test
Korda and Brewinska, (1977a, 1977b)	Instantaneous and scan sampling	2-4 and 13-16	14-hour period with constant sampling throughout, on postnatal day 1-4 and 13-16	1. The amount of time spent by the bitch in direct tactile contact with the puppies 2. Number of acts of licking the puppies by the bitch 3. Number of feeding acts 4. Total time of feeding 5. Puppy feeding posture
Wilsson (1984)	Ad libitum Focal-animal sampling	21-56	15-minute periods × 4 times a day between 0730 and 1200 hours, 5-7 days per week	1. Nursing duration 2. Growling of dam to puppy 3. Inhibited bites (intention to bite the puppy) 4. Nibbling of puppies 5. Licking puppies 6. Pawing at puppies 7. Submission of puppies
Grant, 1987	Instantaneous and Scan sampling	1-21	One hour per day spread across a 24-hour period on days 1-4, 7-10, and 12-21	1. Total time feeding 2. Feeding frequency 3. Average length of feeds 4. Time bitch spent away from puppies 5. Puppy cleaning frequency 6. General observations (bitch position while feeding, puppy action while feeding, cleaning)
Malm and Jensen, 1997	Instantaneous and scan sampling	14-56	48 hours of continuous video recording each week, and 8 × 30 minutes per day on 2 consecutive days each week	Mother behavior: 1. Dam within whelping box 2. Nose contact 3. Licking 4. Growling 5. Inhibited bitePuppy behavior: 6. Suckling 7. Mothers position when nursing 8. Social contact with the mother either through nose contact or begging 9. Teat seeking 10. Sucking outside of regular sucklings 11. Jumping at wall 12. Whining toward mother
Nagasawa et al., 2014	Instantaneous and scan sampling	21-42	Every half an hour for a whole day, once a week	1. Duration of dam lactation 2. Duration of mother licking her puppies
Guardini et al., 2015	Instantaneous and scan sampling	1-21	15-minute video recording	1. Contact mother to puppies 2. Nursing 3. Licking 4. Licking anogenital area
Foyer et al., 2016	Focal-animal	1-21	Every second hour, for one hour, once a week	1. Mother in box 2. Lying on contact 3. Nursing 4. Licking 5. Sniff/poke

2003). Factors such as litter size, weaning weight, and sex ratio of the litter were not relevant in determining the mother's maternal behavior (Champagne et al., 2003). Of pertinence to this article, is that variation in maternal behaviors, such as licking, grooming, and nursing position, have profound effects on the offspring behavioral and neural phenotypes (Pan et al., 2014).

Effect of maternal care in rodents

As the rat and dog are multiparous and altricial species and their developmental stages are similar, it is likely that the relationship between stress and maternal behavior is also similar.

The hypothalamic-pituitary-adrenal axis

A difference in the amount of maternal licking and grooming received alters the offspring's response to novelty. Offspring with high LG-ABN mothers had a significantly reduced fear response to novelty compared to those offspring of low LG-ABN mothers (Caldji et al., 1998). Pups licked more during PNDs 0-10 showed a number of physiological differences including reduced plasma adrenocorticotropic hormone (ACTH), reduced corticosterone responses to acute stress, increased hippocampal glucocorticoid receptor messenger RNA expression, enhanced glucocorticoid feedback sensitivity, and a decreased level of hypothalamic corticotropin-releasing hormone messenger RNA (Liu et al., 1997; Caldji et al., 1998). Some pathways can be seen in Figure 1. Physiological behaviors were positively associated with maternal licking ($r > 0.6$) suggesting that maternal behavior can alter the hypothalamic-pituitary-adrenal response to stress in the offspring (Liu et al., 1997).

Receptors and receptor binding in the central nervous system

Licking and grooming behaviors of the mother alter a major neurotransmitter in the central nervous system, the $GABA_A$ receptor complex (Caldji et al., 2000). The complex is involved with binding molecules, such as benzodiazepines, which have antianxiety effects, suggesting that animals with higher levels of receptor binding are in a lowered stress state for a longer period compared to those who have lower receptor binding. Adults of high LG-ABN mothers had significantly higher levels of the $GABA_A$ receptor binding, and interestingly, binding was not reversible in these animals throughout their lifespan (Caldji et al., 2000). Levels of $\alpha1$ and $\gamma2$mRNA, involved in the functional benzodiazepine-binding site described previously, are altered by maternal care (Caldji et al., 2000). Adult rodents, which were licked and groomed more when young, have increased levels of $\alpha1$ and $\gamma2$mRNA in the amygdala and the locus coeruleus within the brain (Caldji et al., 2000). High LG-ABN mothers may produce offspring with altered mRNA levels in the amygdala, ultimately affecting the mRNA subunits which form the GABA receptor and therefore producing offspring presenting with less anxious behavior as there are higher levels of receptor binding. In addition to this, benzodiazepine receptor density is higher in offspring of high LG-ABN litters (Caldji et al., 1998), allowing for more binding to occur therefore rendering the pup less anxious for a longer period. Moreover, an increase in $\alpha2$ adrenoreceptor density is increased in offspring of high LG-ABN litters (Caldji et al., 1998). This receptor is important as it mediates synaptic transmission of nerve terminals and therefore alters noradrenaline production, reducing the physiological changes (e.g., increase in heart rate) associated with stress (Park et al., 2013).

Production of stress hormones

It has been suggested that oxytocin has antianxiety effects and therefore promotes maternal care, as opposed to CRH which promotes anxiety and disrupts maternal behavior (Uvnas-Moberg, 1997). Variations exist in the oxytocin receptor levels when comparing low and high LG-ABN mothers (Francis et al., 2000). There are sex differences in oxytocin and vasopressin (hormones associated with anxiety and social behaviors), where adult females of high LG-ABN mothers had an increase in oxytocin receptor binding, which was not seen in the adult males. In contrast, the adult males of high LG-ABN mothers had an increase in vasopressin receptor binding in the amygdala, whereas no difference was seen in the females (Francis et al., 2002). An increase in vasopressin binding suggests that the individual is less likely to produce a large quantity of stress hormones, as the vasopressin receptor is also related to adrenocorticotropic hormone (ACTH) release. It is

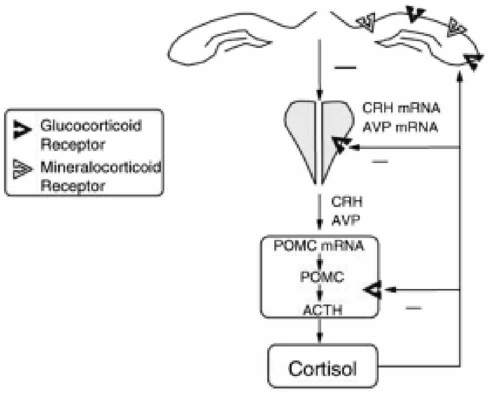

Figure 1. The hypothalamic-pituitary-adrenal axis. Within the paraventricular nucleus of the hypothalamus, corticotropin-releasing hormone (CRH) and arginine vasopressin are expressed and released in blood vessels. There hormones stimulate ACTH resulting in glucocorticoid release from the adrenal gland. Multiple steps within this pathway can be altered by the frequency and duration of licking/grooming and nursing posture. Modified from Kapoor et al. (2006).

interesting that differences in maternal care may alter adult offspring differently, dependent on their sex.

Spatial learning and memory

Differences in the latency and swim path of rodents in the Morris water maze test across days have been documented. This maze test is the gold standard measure of spatial learning and memory in rodents (Milner et al., 2014). There was significantly more searching in the target quadrant, indicating high exploratory behavior, in adult offspring of high LG-ABN mothers compared to low LG-ABN mothers (Liu et al., 2000). Offspring of high LG-ABN mothers also showed a significantly shorter latency and swim path to the target platform compared to adults of low LG-ABN mothers (Liu et al., 2000). This is likely due to differences observed in hippocampal development and in particular the expression of genes encoding for N-methyl-D-aspartate receptor subunits which are important for spatial learning and memory (Liu et al., 2000). It is therefore probable that offspring of low LG-ABN mothers will have detrimentally altered memory and learning capacity as they would have lowered expression of genes encoding for N-methyl-D-aspartate receptor subunits (Morris et al., 1982; Milner et al., 1998; Whishaw, 1998; Wood et al., 1999). Hence, this would indicate that offspring of low LG-ABN mothers would be more anxious, and therefore less likely to explore their environment, as well as offspring having altered learning capacity and memory capability. Caldji et al. (1998) provide further evidence that offspring of low LG-ABN mothers have increased anxiety and neophobia. Offspring of low LG-ABN mothers have a decrease in benzodiazepine and $\alpha2$ adrenoreceptor density (receptors involved in stress hormone production) along with an increase in CRH (Caldji et al., 1998). In a novel environment, this resulted in an increased startle response, a decrease in open-field exploration, and a longer latency to eat food provided (Caldji et al., 1998).

Laboratory practices and rat development

Short separation from the mother, including maternal separation (3-12 hours) and deprivation ($\leq$24 hours) during PNDs 0-14 can alter stress responses in offspring later in life. Short separation of the pup and mother is followed by an increased amount of licking of the pup when it is replaced back within the litter (Lee and Williams, 1974; Lee and Williams, 1976; Anisman et al., 1998). Benefits of additional licking include an increased brain plasticity (plasticity allows the individual to change their body's physiology and behavior in different circumstances) and lower anxiety compared to those pups which were not removed (Liu et al., 1997; Liu et al., 2000). Interestingly, separating the mother and pup for only as short a time as three hours may have detrimental effects on the offspring. Offspring separated from the mother for three hours displayed an increased response to stress, increased anxiety, and cognitive impairment (Lippmann et al., 2007). Longer periods of separation of the mother and pup are also associated with decreased exploration, behavioral inhibition (avoidance behavior), increased CRH mRNA in the paraventricular nucleus, decreased levels of glucocorticoid receptor mRNA in the hippocampus, and an increase in corticosterone response to stress (Plotsky and Meaney 1993; Meaney et al., 1996; Lehmann et al., 1999). Female pups separated from the mother for five hours a day during preweaning displayed a reduced amount of maternal licking and grooming with their own subsequent litter (Fleming et al., 2002). This indicates an intergenerational effect, which may result in epigenetic ramifications.

Artificial and maternal rearing of pups have been compared. To imitate maternal anogenital licking, pups in artificial rearing (AR) groups were stimulated with a soft wet paintbrush over the anogenital region. For the minimum AR group, each pup was swabbed in the anogenital region twice a day for 30 seconds. In the maximum AR group, each pup was swabbed in the anogenital region twice a day for 30 seconds each time plus had overall body stroking 8 times a day for a total of two minutes beginning at PND three or four until PND 16 (Lovic et al., 2006). The artificially reared groups were more active in response to novelty tests compared to the mother reared groups (Lovic et al., 2006) which may indicate increased state of arousal due to distress. In support of this interpretation of the finding of Lovic et al. (2006), Gonzalez and Fleming (2002) determined that AR groups are more likely to react negatively during experiences compared to those pups reared by their mothers. Physiologically, there was a significant reduction in c-fos immunoreactivity in the medial preoptic area and the parietal and piriform cortices, areas involved in stress and pain measurement. Results of these studies (Gonzalez and Fleming, 2002; Lovic et al., 2006) may be due to methodology used. Gonzalez and Fleming (2002) and Lovic et al. (2006) both had two groups of artificially reared pups one group had a minimum amount of stimulation for urination and defecation, whereas the other group had a longer duration of stimulation. However, the frequency and duration of stimulation differed between the studies. In the Gonzalez and Fleming (2002) study, the minimum AR group received two swabs per day lasting for a total of 45 seconds while the maximum AR group received five swabs per day lasting for a total of 2 minutes. This process lasted for 16 days (Gonzalez and Fleming, 2002). In contrast, in the study conducted by Lovic et al. (2006), each pup in the minimum AR group was stimulated twice a day for a total of 30 seconds. In the maximum AR, each pup received eight stimulations per day for a total of two minutes. Each group experienced stimulation for between 13-14 days. The duration of nursing, and the posture undertaken by the mother when feeding was not considered in these articles. Some consideration is needed when stating the resulting effect is from stimulation alone. In addition, both of the aforementioned studies completely separated the pups away from both the litter and the mother and therefore were reared as isolated individuals. When the pups remain within the litter and the mother is merely separated, novelty induced locomotion was not altered (Matthews et al., 1996 and Brake et al., 2004). This has identified that the separation duration along with the separation method (whether the pup remained within the litter or remained alone) is important in determining the response the offspring will have later in life.

Interspecific differences between the dog and the rat

The gestation of the rat is approximately 21 days (Eleftheriades et al., 2014), and frequently, the mother is both lactating and pregnant at the same time (Prager et al., 2010). The domestic dog has a normal gestational period between 64-66 days (Wells and Hepper, 2006) and the bitch comes into estrous approximately every seven months (Macdonald and Carr, 1995). However, the age the bitch first comes into estrous differs with breed size; small females (8-15 kg) come into season at seven months of age while larger females (30-40 kg) can come into season as late as 18 months (Lord et al., 2013).

Dogs and rats are comparable in that they both, usually, have more than one offspring in their litter. Rat litters range from 6-12 pups (Viana et al., 2013) while in seven purebred breeds with over 1,00,000 litters recorded, average litter size ranged from 3.5 ± 1.7 to 6.3 ± 3.1 puppies (mean ± standard deviation) (Leroy et al., 2015). Using a smaller number of litters (10,810) but a larger variety of breeds, an average overall litter size at birth was calculated to be 5.4 $\pm$ 0.025 puppies, with a range of 1-18 puppies (litter size included puppies born both alive and dead) (Borge et al., 2011).

Myelin and the myelin sheath around axons of neurons are important in promoting neural conduction in the nervous system (Simons and Lyons, 2013). Development of myelin sheath differs between mammals. In dogs, the brain showed signs of myelin fibers in the posterior fossa at two weeks of age while secondary myelin branching was apparent after 1.5 months of age (Moit-Noirault et al., 1997). In comparison, myelin sheath was present within three days of birth in the rat and developed rapidly until the pup was two weeks old (Webster, 1971). In both species, this neural development period overlaps with the preweaning period of the litter. Thus, it is likely the significant effects that maternal care has on rat pups are also likely to occur in dog puppies.

Critical events in the first two weeks of offspring life in the rat and the dog are compared in Table 2. Maternal care is crucial within the first two weeks as the offspring requires their mother for

Table 2
Critical events in the first 2 weeks of life of dogs and rats

Developmental event	Rat (PND)	Dog (PND)
Eyes open	14-15[a]	13[b]
Hearing	9[c]	19[b]
Smelling	By day 1[d]	Respond to chemosensory cues from birth[e,f] Fully functional between 8-13[g]
Independently urinating and defecating	Mothers lick at high rates until 21 days, suggesting this is when they begin to undertake process independently[h]	14-21[i]

Values are days postnatal (PND).
[a] Weisse, 1992.
[b] Feldman, 1992.
[c] Méndez-Gallardo and Robinson, 2011.
[d] Moore and Chadwick-Dias, 1986.
[e] Battaglia, 2009.
[f] Fox, 1971.
[g] Wells and Hepper, 2006.
[h] Scott et al., 1974.
[i] Lawler and Chandler, 1992.

survival. The critical events in the rat and the dog offspring occur relatively close to one another, with the exception of hearing. This is therefore additional evidence to justify comparing early environment and later stress response from the rat to the dog.

Conclusion

Differences in maternal behavior alter the offspring later in life, in particular behavior and physiological responses to stress. Rodents may provide a useful maternal care model for dogs in that both species are litter-bearers and altricial. Naturally occurring differences in maternal care behaviors exist in rats and have been studied extensively. Mothers which are classified as low licking/grooming and arched-back nursing may cause detrimental effects in their offspring and change the HPA axis causing an increase in the offspring's stress response. These physiological differences are reflected in behavioral changes in the offspring such as an increase in startle response, a decrease in exploration, a longer latency to eat, a longer latency to reach a selected target, and a longer swim path to a selected target. However, maternal care behaviors in the dog still need to be accurately documented within the first two weeks of life. Describing differences and classifying dams on their maternal care will have significant implications for dog welfare. As the number of dogs relinquished to shelters for undesirable behavior, in particular anxiety/stress and separation anxiety, is high, there is a need to understand early environments which may be causing welfare problems later in the dog's life. Furthermore, given that such early experience can have an intergenerational effect, knowledge of maternal behavior in domestic dogs has implications for breeding plans where the selection of a more appropriate dam may lead to offspring which have a lowered stress response.

Conflict of interest

There is no conflict of interest.

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Appendix 3: Published Journal Article 2

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Sampling maternal care behaviour in domestic dogs: What's the best approach?

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ABSTRACT

Our understanding of the frequency and duration of maternal care behaviours in the domestic dog during the first two postnatal weeks is limited, largely due to the inconsistencies in the sampling methodologies that have been employed. In order to develop a more concise picture of maternal care behaviour during this period, and to help establish the sampling method that represents these behaviours best, we compared a variety of time sampling methods Six litters were continuously observed for a total of 96 h over postnatal days 3, 6, 9 and 12 (24 h per day). Frequent (dam presence, nursing duration, contact duration) and infrequent maternal behaviours (anogenital licking duration and frequency) were coded using five different time sampling methods that included: 12-h night (1800–0600 h), 12-h day (0600–1800 h), one hour period during the night (1800–0600 h), one hour period during the day (0600–1800 h) and a one hour period anytime. Each of the one hour time sampling method consisted of four randomly chosen 15-min periods. Two random sets of four 15-min period were also analysed to ensure reliability. We then determined which of the time sampling methods averaged over the three 24-h periods best represented the frequency and duration of behaviours. As might be expected, frequently occurring behaviours were adequately represented by short (one h) sampling periods, however this was not the case with the infrequent behaviour. Thus, we argue that the time sampling methodology employed must match the behaviour of interest. This caution applies to maternal behaviour in altricial species, such as canids, as well as all systematic behavioural observations utilising time sampling methodology.

1. Introduction

The selection of time sampling method is critical for accurately describing the frequency and duration of behavioural traits (Altmann, 1974; Daigle and Siegford, 2014). Ideally, the measurement period should be continuous in order to ensure that every event is measured within that period. However, continuous monitoring over long periods of time is rarely practical, with researchers often opting to measure behaviour across intervals. It is important that the frequency and duration of the behaviour of interest be ascertained prior to the start of any study (Altmann, 1974), as different time periods are required to identify specific behaviours (Daigle and Siegford, 2014). That is, the relative magnitude of the sampling error will depend on the frequency of the behaviour, its duration, and the number of observations made over the sampling period (Powell et al., 1977; Saudargas and Zanolli, 1990). In particular, infrequent behaviours or those with short durations can be erroneously measured if a short time sampling method is used (Saudargas and Zanolli, 1990). The importance of time sampling methodology and its implications have been documented and compared in other species (e.g. laying hens: Daigle and Siegford, 2014; rodents: Saibaba et al., 1996), but not so in the domestic dog. Although the principle of, and consequences of sampling methods extend across most species, it is uncertain how the methodology used to date impacts our understanding of maternal care in dogs.

A number of maternal care behaviours in domestic dogs have been explored during the first few weeks after birth. The behaviours documented are specific to the duration, and at times the frequency, of dam presence, nursing, contact and anogenital licking (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a,b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016; Guardini et al., 2016). However, it is difficult to compare the findings of these studies due to inconsistent behavioural definitions and measurements that have been used (Table 1). For instance, the way in which contact between dam and puppy has been measured varies across studies. Rheingold (1963) defined contact as any physical contact between the mother and pups, while Foyer et al. (2016) recorded contact as the duration of time the

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Table 1
Comparison of studies describing maternal care behaviours (dam presence, nursing, contact and licking) in dogs within the first 12 postnatal days.

Study	Breed (no.) and dams observed	No. of puppies	Sampling method	Sampling time (day/night)	Sampling range (postnatal days)	Total observations (hrs)
Rheingold, 1963	Cocker Spaniel (1) Sheltie (3) Beagle (1)	24	Average of 4 × 15 min each day	0800–1800 h (day)	1–68	153
Scott and Fuller, 1965	Basenji (4) Beagle (5) Cocker Spaniel (5) Sheltie (5) Fox terrier (5)	DNS	10 min weekly observations	DNS ('daily')	1–28	20
Korda and Brewinska, 1977a, 1977b	Control: Mongrel (4)	32–36	14 h daily continuous sampling	DNS ('daily')	2–4 and 13–16	784–882
Grant, 1987	Beagle (1)	7	One hour per day across a 24-h period, on days 1–4, 7–10, and 12–21	24 h (day and night)	1–21	18
Guardini et al., 2015	Weimeraner (1) Belgian Shepherd Groenendael (3) Cross-bred (1) Short Haired Dachshund (1) German Shepherd (1) Labrador retriever (1) Boxer (1) Border Collie (1)	58	15-min video recording	Morning (day)	1–21	52.5
Foyer et al., 2016	German Shepherd (22)	94	Every second hour (12 h) on one day, once a week	24 h (day and night)	1–21	1056
Guardini et al., 2016	Beagle (8)	54	15 min once a day, every day for the first three postnatal weeks	Morning (day)	1–21	5.25

DNS = Data not shown.

Table 2
Comparison of studies reporting maternal care behaviour in dogs within the first twelve postnatal days. The range in the duration of a behaviour reported reflects differences between dams.

Behaviour observed	Behaviour duration (%)
Dam presence	20–100[a,g]
	58–81[b]
	60–98[c,g,h]
Nursing	3–84[a,g]
	13–83[d]
	82–99[e,g]
	20–63[b,g]
	0–100[f,g]
	8–23[c,g,h]
Contact	10–100[a,g]
	29–86[e,g]
	10–32[c,g,h]
Licking	0.1–8.9[a,g]
	14–61 acts[b,g]
	0–100 acts[e,g]
	0–33.9[f,g]
	1–5[c,g,h]

[a] Rheingold (1963).
[b] Grant (1987).
[c] Foyer et al. (2016).
[d] Scott and Fuller (1965).
[e] Korda and Brewinska (1977a,b).
[f] Guardini et al. (2015).
[g] Behaviours were determined from figures only (no information provided in text).
[h] Behaviours were averaged per litter size and therefore behaviours are presented per puppy.

mother has elbows on the ground and is in physical contact (excluding the tail and limbs) with at least one puppy within the whelping box. There are also differences in the methodologies employed for quantifying maternal behaviours. For example, anogenital licking has been measured both as a frequency (Korda and Brewinska, 1977a; Grant, 1987) and duration (Rheingold, 1963; Guardini et al., 2015; Foyer et al., 2016). As Table 2 demonstrates, the sampling methodologies used across various studies has resulted in variation in the reported duration of the behaviour. To date, studies focusing on maternal care only describe the dam when she is with the entire litter (Rheingold, 1963; Scott and Fuller, 1965; Korda and Brewinska, 1977a,b; Grant, 1987; Guardini et al., 2015; Foyer et al., 2016; Guardini et al., 2016), which ignores the interactions between dam and individual puppies. Thus, our understanding of the frequency and duration of maternal care behaviours in dogs remains relatively uncertain.

In this study, we aimed to determine whether time sampling methodology influences the reporting of maternal care behaviours in domestic dogs. We focussed on maternal care exhibited during the first twelve postnatal days, and compared time sampling methods used in previous studies to the frequency and duration of behaviours averaged over three 24-h periods. The rationale for limiting our analysis to the first two weeks post-natal, were two-fold: First, this is the time when the maternal behaviours of interest occur most frequently (Kendrick et al., 1997). Second, maternal care, and in particular anogenital licking, within the neonatal period is important due to the likely influence on offspring stress development and behaviour (Czerwinski et al., 2016). With this in mind, we also aimed to rank puppies according to the frequency in which they are licked (in the anogenital area) by the dam. Doing so will enable us to determine whether the rate that the puppies were licked remained consistent across all sampling methods.

2. Methods

2.1. Animal subjects

Data from six litters of domestic dogs were used (see Table 3). For litters where puppies were difficult to distinguish, a unique collar (i.e. ribbon, paper wrist band) was placed around their necks. This was done with permission from the breeder, and appeared not to disrupt either the dam or the puppy. Breeders were asked not to alter their normal routine, and at no time did the researchers interact with the dam or

Table 3
Dam and litter information.

Litter	Breed	Dam and litter location	Dam parity	Dam age (years)	Litter size (postnatal day 1)
1	English Staffordshire Terrier	Inside house	1	2.5	6
2	Whippet	Inside house	3	6.5	5
3	Greyhound 1	Room outside house	4	8	5
4	Greyhound 2	Room outside house	1	5	3
5	Labrador	Inside house	3	3	1
6	Border Terrier	Inside house	2	3.5	5

puppies. Ethics approval was granted from the University of Adelaide Animal Ethics Committee (S-2014-098).

2.2. *Classification of maternal care behaviours*

Four maternal care behaviours, adapted from Rheingold (1963) and Zahed et al. (2008), were coded using behavioural analysis software (Mangold Interact, v.9). Behaviours included: *dam presence* (any time in which the dam had at least one paw within the whelping box); *Nursing* (any time in which a puppy had its mouth around a teat of the dam. The dam could be sitting, lying on her side or standing); *Puppy in contact with another individual (dam or puppy)* (any time in which a puppy had at least 50% of its body touching the dam or a littermate. This included contact while sitting or lying); and *Anogenital licking* (any movement of the dams tongue along the genitalia or anus of a puppy). Nursing and Puppy in contact with another individual (dam or puppy) were not mutually exclusive behaviours. Based on the frequency in which the behaviours have occurred in previous studies, Dam presence, Nursing, and Puppy in contact with another individual (dam or puppy) were deemed 'frequently occurring behaviours', and anogenital licking was considered an 'infrequently occurring behaviour'.

2.3. *Time sampling methods*

Litters were video-recorded continuously using a fixed surveillance camera (Swann PRO-530) mounted in a position that overlooked the whelping box. Video was recorded at 20 frames per second with 1024 maximum bitrate (Kbps) using a digital video recording device (4/8 Channel D1 Realtime H.264 DVR). Recording occurred from postnatal day 0 until postnatal day 13, except for Litter 4 which was recorded from postnatal days 5–13. Each of the litters were coded for 96 h over postnatal days 3, 6, 9 and 12 (24 h per day), with the exception of Litter 4, which was coded for 72 h over postnatal days 6, 9 and 12.

The following sampling methods were used: 24-h: Each of the four post-natal days were coded continuously for the 24-h period. The average of these three 24-h periods were used as the 'gold standard' method to which the following time sampling methods were compared. 12-h *night* and 12-h *day*: The 24-h periods were split into two 12-h periods covering the night (1800–0600 h) and the day (0600–1800 h). *One-hour anytime (set 1 and 2)*: Four random 15-min periods were selected from each 24-h period using the 'Random Number Generator' mobile phone application (Skytrait, V 4.2.0). The four 15-min periods were then combined to equal one hour. A second set of 15-min periods were randomly selected for each of the one-hour sampling methods to ensure repeatability of the measure when a different random selection were chose. *One-hour night (set 1 and 2)* and *One-hour day (set 1 and 2)*: Four random 15-min periods were selected from the night (1800–0600 h), and during the day (0600–1800 h).

2.4. *Statistical analysis*

Anogenital licking was measured using frequency. The frequency data were standardised per hour to allow comparison between sampling methods. Dam presence, nursing and puppy in contact with another individual (dam or puppy) behaviour were measured in time, and calculated as a percentage of the total time the dam was present in the whelping box to account for differences in recording times. Puppies were also ranked across days for each sampling method to allow differences in ranking to be described. The litters were ranked for each behaviour from highest to lowest across each observation day for anogenital licking only. We focused on anogenital licking because of its likely importance on future stress behaviour in puppies (Caldji et al., 1998; Czerwinski et al., 2016), and to determine how sampling methodology influences the measurement of infrequently occurring behaviours.

Data for the four behaviours were analysed using a multivariate general linear model in SPSS (IBM, v.21) to determine whether there was a difference in frequency or duration between the time-sampling methods. Normality tests determined all behaviours (dam presence, nursing, contact, anogenital licking) were not normally distributed and therefore the data were log transformed. Post hoc tests, using Least Square Differences, were used to determine significance between time sampling methods. The Wilcoxon Signed Ranks Test was used to determine differences in puppy ranking in regards to anogenital licking between 24-h, 12-h night and 12-h day across the four coding days. Significance was accepted at $p < 0.05$.

3. Results

3.1. *Frequently occurring behaviours*

3.1.1. *Dam presence*

The amount of time the dam spent within the whelping box significantly differed between the 24-h period and the time sampling methods (F (8, 113) = 2.50, p = 0.015; partial η^2 = 0.15). The 12-h day time sampling method resulted in a significant reduction in dam presence compared to the 24-h period (Fig. 1A). There was no significant difference between the one hour periods when comparing random set 1 and random set 2 for the night, day, or anytime period ($p > 0.05$).

3.1.2. *Nursing*

There were no significant differences (F (8, 113) = 0.82, p = 0.585; partial η^2 = 0.06) between the 24-h period and any of the time sampling methods (Fig. 1B). Both one hour random set 1, and random set 2 (for night, day and anytime periods), did not significantly differ to one another ($p > 0.05$).

3.1.3. *Puppy in contact with another individual (dam or puppy)*

There were no significant differences (F (8, 113) = 1.46, p = 0.179; partial η^2 = 0.09) between the 24-h period and any of the time sampling methods for contact behaviour (Fig. 1C). No significant differences were found between the random set 1 and random set 2 for the short time sampling methods (one hour night, day and anytime periods).

3.2. *Infrequently occurring behaviour*

3.2.1. *Anogenital licking*

Differences were observed between the 24-h period and time

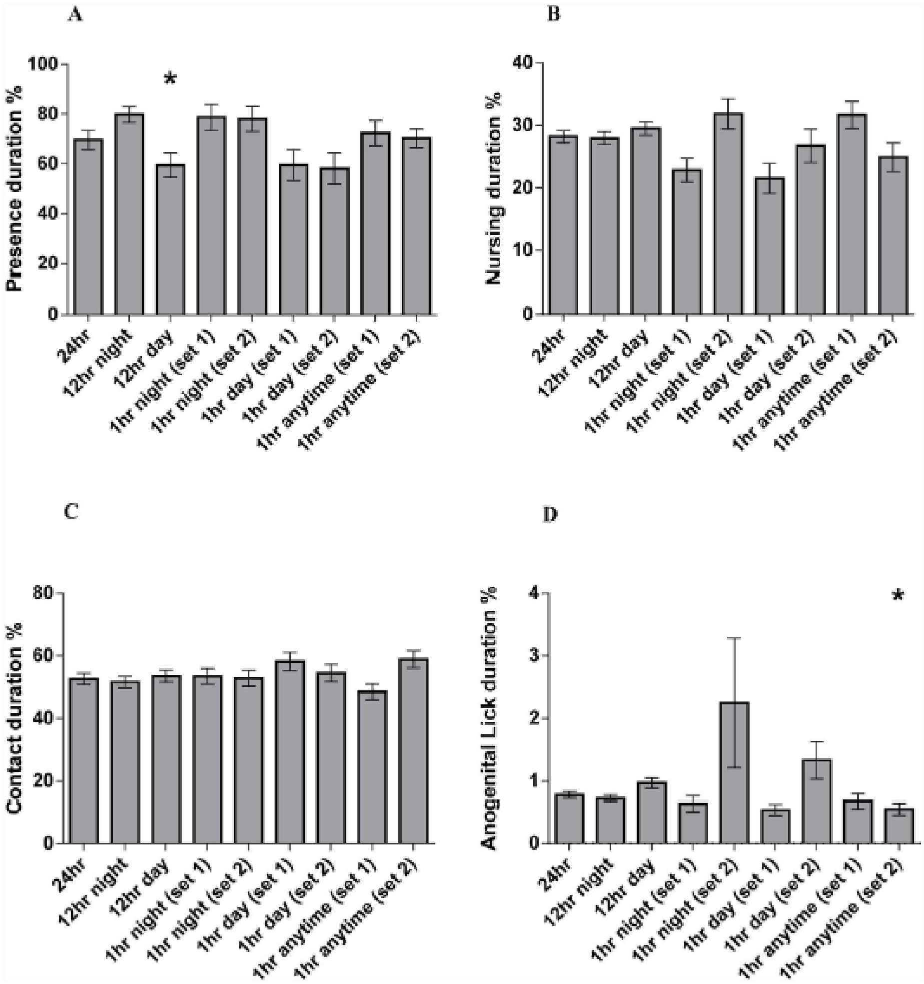

Fig. 1. Comparison of time sampling methods for A) Dam Presence, B) Nursing C) Puppy in contact with another individual (dam or puppy) and D) Anogenital Licking. Values are expressed as a mean value of the four coding day's ± standard error of the mean. * indicates a significant difference to the 24-h period when comparing log transformed data.

sampling method (F (8, 113) = 2.04, p = 0.048; partial η^2 = 0.13). The one hour anytime (set 2) time sampling period showed a significantly higher amount of anogenital licking compared to the 24-h period (Fig. 1D). Although there was a significant difference between one hour anytime random set 1 and set 2, there were no significant differences between one hour random set 1 and set 2, or between the one hour night and one hour day periods ($p > 0.05$). In regards to anogenital licking frequency, both random set 1 and set 2 for the one hour time sampling methods (night, day and anytime) significantly underestimated anogenital licking compared to the 24-h period (Fig. 2).

There were no significant differences in ranking results when the 12-h night (Z = −0.339, p = 0.735) and the 12-h day (Z = −0.222, p = 0.824) time sampling methods were compared to the 24-h period ($p > 0.05$). However, all six variations of the short time sampling methods (one h) were significantly different to the 24-h period ($p < 0.05$).

4. Discussion

The aim of the study was to determine which time sampling method was optimal for measuring the frequency and duration of maternal care behaviour in domestic dogs. To achieve this, we compared the behaviours using a variety of time sampling methods to a 24-h period. We determined that the accuracy of the observations related to the time sampling method used. That is, longer time sampling methods (e.g. 12-h day and 12-h night) can be used in lieu of 24-h period for some frequently occurring behaviours such as nursing and contact behaviour, and a 12-h night time sampling period representative of dam presence. In fact, a time sampling method as short as one-hour (consisting of four 15-min periods, randomly selected during the day, night or anytime throughout the 24-h period), may be used for some frequently occurring behaviours across a 24-h period.

For infrequent behaviours such as anogenital licking however, the use of short time sampling methods do not accurately reflect the frequency or duration when compared with a 24-h period. Further,

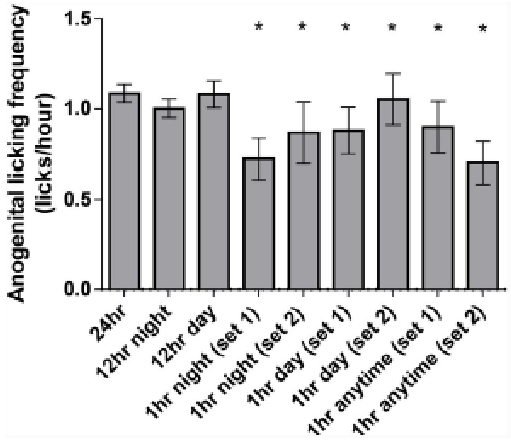

Fig. 2. Comparison of different time sampling methods for frequency of anogenital licking per hour. Values are expressed as a mean of the four coding day's ± standard error of the mean. An asterisk above a sampling period indicates a significant difference to the 24-h period when the data were log transformed.

when short time sampling methods were used to measure anogenital licking in the current study, the results were highly inconsistent. This finding is not unexpected, as several studies have reported that short time sampling methods do not accurately describe infrequent and low duration behaviours (Powell et al., 1977; Saudargas and Zanolli, 1990). Rheingold (1963) and Guardini et al. (2015) have previously explored anogenital licking, but given their sampling methodology, it is likely that their results do not accurately represent the true behaviour. Accurately describing the frequency of behaviours is critical, as for example, the amount of anogenital licking a pup receives may in fact alter its brain development and their response to stressful situations later in life (see Czerwinski et al., 2016).

In observational studies of maternal care behaviour in dogs, it is common for the behaviours to be recorded for the entire litter and hence coded from the perspective of the dam. This presents the opportunity to gain a true picture of the behaviour of the whole litter, but prevents the ability to detect individual differences in puppy behaviour, or the interaction between the dam and puppies. For example, when coding at the litter (or group) level, it is impossible to distinguish how many, or which, individual puppies are feeding. Within the current dataset, puppies were individually identified, and their behaviours recorded (with the exception of dam presence). We found that dams engaged in anogenital licking between 0.09–4.10% of the time when observed for a 12-h period. This was similar to the range (1–5%) observed by Foyer et al. (2016). However, in that study, the duration of anogenital licking was estimated across litter size and they were not able to distinguish individual puppies consistently. It is highly likely that dams do not provide equal care among all her offspring, and thus, the development of each puppy might differ as a result.

Within the current study, we found no difference between the 24-h period and the 12-h periods for the frequency of anogenital licking (either across the night or day), suggesting that 12 continuous hours coded either day or night, is representative of the behaviour across a 24 h period. However, it is likely that the definition of anogenital licking is more influential than the sampling method. For example, Korda and Brewinska (1977a) documented between 30 and 100 licking acts per litter within the first twelve days postnatal, while we reported a much smaller range (0–41 licks per litter). In our study, each licking event was recorded regardless of the duration in which it occurred, whereas Korda and Brewinska (1977a) recorded anogenital licking events with a duration over 30 s, and as a result, one licking event could include multiple puppies.

To further understand sampling methodology and its accuracy, it is important to determine the number of hours over which an infrequent behaviour can be determined. It may be possible that a time period anywhere from one to 12 h can provide representative results. To gain a more accurate picture of infrequent behaviours, a more selective period may improve the likelihood of capturing the behaviour. Guardini et al. (2016) for example, coded behaviour for 15 min after the dam had returned to the litter in the morning. This is likely to capture a high level of activity, given the separation period of the mother from the litter can stimulate a greater expression of maternal behaviour when the dam returns. Further, the dogs in our study represented a number of breeds, dams differed in relation to maternal experience (parity), and the size of each litter was variable. The true impacts of each of these aspects are currently uncertain, and should be investigated further. Lastly, our data came from dogs living in the breeder's household (which comes with potentially distracting human daytime activity), where some of the previous studies reporting less variance were measured in presumably more constant laboratory situations. Thus, we also recommend that future studies observe the interaction of the handler/breeder on the litter to determine the impact they may have on the dam's behaviour.

5. Conclusion

We found evidence to suggest that not all behaviours need to be observed for a full 24-h period to get an adequate representation of frequency or duration. Short time sampling methods can accurately represent a 24-h period, but only for frequently occurring behaviours. These include: dam presence, nursing, and lying in contact with another individual (dam or puppy). For the assessment of low-frequency behaviours, such as anogenital licking, short time sampling methods (e.g. one-hour duration), are unsuitable and in these situations, a longer, continuous period of time of observation is recommended. These findings highlight the influence of time sampling on behaviour representations, and provide useful insight for future studies measuring maternal care in canids, as well as in other altricial species.

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Appendix 4: Published Journal Article 3

animals

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Article

Selection of Breeding Stock among Australian Purebred Dog Breeders, with Particular Emphasis on the Dam

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Simple Summary: One of the most important factors influencing the health and welfare of puppies is the decision made by the breeder on which dam and sire they will breed from. Unfortunately, our understanding of what dog breeders consider important when selecting their dogs, particularly the dam, is limited. In order to bridge this gap, we conducted an online survey of Australian purebred dog breeders. We identified four major factors that the breeder considered important in relation to the dam: Maternal Care; Offspring Potential; Dam Temperament; and Dam Genetics and Health. Overall, the priorities and practices of dog breeders surveyed were variable across breeds. Importantly, it seemed that not all breeders understood the importance of maternal care behaviour, despite the significant role it may play on future puppy behaviour.

Abstract: Every year, thousands of purebred domestic dogs are bred by registered dog breeders. Yet, little is known about the rearing environment of these dogs, or the attitudes and priorities surrounding breeding practices of these dog breeders. The objective of this study was to explore some of the factors that dog breeders consider important for stock selection, with a particular emphasis on issues relating to the dam. Two-hundred and seventy-four Australian purebred dog breeders, covering 91 breeds across all Australian National Kennel Club breed groups, completed an online survey relating to breeding practices. Most breeders surveyed (76%) reported specialising in one breed of dog, the median number of dogs and bitches per breeder was two and three respectively, and most breeders bred two litters or less a year. We identified four components, relating to the dam, that were considered important to breeders. These were defined as Maternal Care, Offspring Potential, Dam Temperament, and Dam Genetics and Health. Overall, differences were observed in attitudes and beliefs across these components, showing that there is variation according to breed/breed groups. In particular, the importance of Maternal Care varied according to dog breed group. Breeders of brachycephalic breeds tended to differ the most in relation to Offspring Potential and Dam Genetics and Health. The number of breeding dogs/bitches influenced breeding priority, especially in relation to Dam Temperament, however no effect was found relating to the number of puppies bred each year. Only 24% of breeders used their own sire for breeding. The finding that some breeders did not test for diseases relevant to their breed, such as hip dysplasia in Labrador Retrievers and German Shepherds, provides important information on the need to educate some breeders, and also buyers of purebred puppies, that screening for significant diseases should occur. Further research into the selection of breeding dams and sires will inform future strategies to improve the health and behaviour of our best friend.

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Keywords: dog breeding; purebred dog; survey; maternal care

1. Introduction

There are an estimated 4.2 million dogs within Australia [1] with most dogs bred for the purpose of companionship [2]. In Australia, the Australian National Kennel Club (ANKC) is the registered organisation for pedigree dog breeders. In 2015, its 32,481 members, 20% of whom were active breeders, produced (and registered) 69,274 puppies [3]. Given that it is impossible to determine the number of puppies born to non-registered breeders, the total number of puppies born in Australia each year is unknown.

One of the primary aims of the ANKC is to provide members with breed standards that promote behaviourally and physically sound dogs for ownership, as well as promoting excellence in a number of dog-related fields, such as breeding, showing, trialling, obedience and other canine related behaviour. Although the ANKC collects information about registered breeders (i.e., number of breeders per breed, number of active breeders, number of litters produced per year, number of puppies produced) through the state bodies, data collected does not extend to the breeding priorities and practices of the breeders. This lack of information extends to the scientific literature, with little known about dog breeding practices and philosophies in Australia for registered and non-registered breeders. Such information is vital for improving breeding practices, and ensuring the optimal health and behaviour of dogs.

With over 200 breeds registered with the ANKC, breeding practices are likely to be as diverse as the breeds themselves [4]. For example, the purpose for which the dog is bred (i.e., companion, working) is likely to be reflected in the way the dogs are housed and bred. Often, the most important aspect of pedigree or pure breeding involves the selection of breeding animals that conform to a set standard [5], which is usually determined by a registered organisation such as the ANKC. Physical characteristics (e.g., body conformation, coat length and colour, height, facial appearance, gait), as well as certain behaviours (e.g., instincts such as herding, hunting or retrieving, temperament and trainability) are taken into consideration when choosing breeding stock [6]. Priorities of breeders are also likely to alter over time. For instance, in the past, dogs were primarily bred for various working roles, but the focus has shifted to selecting for suitable companion animals, moving towards dog conformation rather than performance [5]. Breed specific diseases are now highly recognised [7,8], allowing for accessible knowledge to be implemented by the breeder. Health risks are also being associated with natural mating, and thus sire selection and mating techniques are also necessary to consider [9,10].

To date, the goals and practices of dog breeders across the world have received little attention. Notable exceptions include a study looking at inbreeding and breed effective population size in an Australian sample of breeders [11], and the selection of dogs and breed goals documented in a French population [4]. In that study [4], 985 French dog breeders, representing 10 different breed groups were asked what considerations they gave to conformation, behaviour, health, work, feeling and reproduction. The behaviour of the dog was considered significantly more important by breeders of sheepdogs, cattle dogs and retrievers compared to all other dog groups. Although the number of litters produced did not significantly alter breed goal, litter production was impacted by breed group; working dogs produced less litters than other breed groups [4]. Leroy et al. [4] also discovered that there were different types of breeders (i.e., occasional, regular hobby and professional breeders) and regular hobby and professional breeders bred from their bitches earlier and therefore had more litters throughout the dam's life. Overall, breeders reported four common goals: (1) dog conformation; (2) behaviour; (3) health and (4) work. Notably, breeders did not consider maternal care as a factor in the selection of breeding bitches [4], despite the importance that it can have on offspring development (e.g., [12–14]). Other factors including the type of birth (i.e., natural vs caesarean), may also affect the dam's behaviour towards the puppies. Caesareans are more likely to occur in certain breeds according to their cranial features [15–17], yet there is no literature on the impact of birth type and maternal behaviour.

Three recent studies have highlighted the importance of maternal care in dog development [18–20]. A correlation was found between maternal care and later anxiety in puppies, with poor maternal care in puppyhood increasing the likelihood of anxiety in dogs, measured using questionnaires [18]. In the second study, maternal care (dam in box, lying in contact, nursing, licking and sniff/poke) observations were undertaken on 22 litters [19]. The dams were observed for the first three weeks postnatal, and then classed as high or low maternal care. By linking maternal care and temperament measured at 15–18 months old, the authors discovered a relationship between the level of maternal care given and physical engagement, social engagement and aggression. An increased interaction between puppy and dam led to adult offspring being more competitive, more engaged in social activities with humans, and with higher aggression levels (as defined by the dog's sharpness and defence drive). The amount of maternal care given to the puppies also alters the behaviour of the puppies when they are 8-weeks-old [20]. In a similar study [20] using an isolation test, puppies that were licked more had an increased amount of exploration and a longer latency to first yelp. Increased licking also reduced the duration in locomotion and time spent interacting with the enclosure, and a shorter duration in vocalisation. These data highlight the influence that maternal care can have on future stress responses in puppies.

Currently, information regarding dam and sire selection by Australian dog breeders remains poor. The objective of this study was to understand factors considered important in the selection of Australian Purebred breeding animals with a focus on factors relating to the dam. It was expected that factors such as ANKC breed group, the number of litters produced and, whether the breed is brachycephalic would impact dam selection. The influence of sire selection and health aspects of breeding were also investigated.

2. Methods

2.1. Survey

We developed a series of questions relating to breeding practices that included questions used by Leroy et al. [4]. Breeders and ANKC members were then consulted (through email and phone) to ensure that the questions, language and terminology were appropriate for Australian dog breeders. The final survey was hosted on Qualtrics (Qualtrics, LLC, Provo, UT, USA), and was available for four months, from March to June 2015. The survey was advertised, with permission, on several online resources frequented by dog breeders. These included: Dogs SA (www.dogssa.com.au); Dogs NSW (www.dogsnsw.org.au); public breeder pages on Facebook; Dogz Online forum (www.dolforums.com.au); Vet answers blog (www.vetanswers.com.au/blog); and the German Shepherd Club of South Australia (www.gsdcsa.org.au). A description of the survey and the survey link was posted onto the researchers (VC) Facebook page and on an online community noticeboard (www.gumtree.com.au), and the survey was broadcast on local Adelaide radio (101.5 FM).

The questionnaire was anonymous and participants were not required to respond to all questions. The survey consisted of 58 questions and took approximately 15 min to complete. There were four questions pertained to the breeder's demographics; 20 questions related to breeding management; 30 questions related to the importance of qualities associated with the dam and the sire; and four questions relating to DNA and physical testing. A full version of the questionnaire can be found in Supplementary Materials. The importance of the dam and sire were represented by multiple questions in a Likert scale where the breeder could rate the importance from Strongly Agree (1) to Strongly Disagree (5). All responses were considered for the first breed listed by the dog breeder, if the breeder bred more than one dog breed. Approval from the University of Adelaide Human Ethics Committee was obtained (H-2014-270).

2.2. Statistical Analysis

2.2.1. Data Transformation

For open-ended responses where a range was given as a response, the average of the range was used. For example, when asked "On average, how often will you breed from each bitch?" an answer of "1–3" was then changed to "2". To describe the physical and genetic tests performed, only those breeds with more than five respondents were included. This allowed satisfactory comparison within the data for types of dog testing. Breeders that bred more than one dog breed were excluded to remove any confusion as to which DNA or physical test was undertaken on which breed. Although some physical and genetic tests are breed specific, many times the response could be applied to several breeds (i.e., X-ray for hip score).

2.2.2. Univariate Analysis of Variance (ANOVA) and PCA

Normality was not achieved in the components (components were positively skewed) and therefore they were log transformed. The Tukey method [21] was used to determine outliers. Firstly, the first and third quartile are identified. The Interquartile range (third quartile minus the first quartile) is then multiplied by 1.5. This value is subtracted from the first quartile and again added to the third quartile. Numbers which have fallen below these values are identified as outliers. Three breeders were removed as they were determined as outliers (Case numbers: 69, 180 and 223), resulting in 271 breeders for results using ANOVA. Twenty-three items relating to the dam were reduced into five components using Principal Component Analysis. However, after observing the matrix, one component was removed leaving four components. Components were analysed using a univariate general model to determine whether there were differences between Australian National Kennel Club dog breed group, number of litters produced, number of breeds the breeder owns and breeds, and brachycephalic dog breed. An eta-squared value (η^2) was calculated; this refers to the effect size (strength between two variables) and is described for each ANOVA. Tukey post-hoc tests were used to determine significance between pairwise comparisons of means. All statistical analyses were performed using SPSS (v.21, IBM, Armonk, NY, USA). Significance was accepted at the 5% level.

3. Results

3.1. Participants

A total of 360 Australian purebred dog breeders completed the survey. However, 86 participants (24%) were discarded due to insufficient responses resulting in a total of 274 (unless stated otherwise). In particular, if any questions regarding the dog breed or dam behaviour were not answered, the respondent's results were removed. A total of 91 dog breeds were represented in the survey, and are described in Table 1. The majority of participants bred Working dogs (21.2%) followed by Gundogs (19.3%), while the Toy group (9.9%) were the least represented (Table 1).

Brachycephalic dog breeds are those which have a facial skeleton relatively shorter than the cranial cavity [17]. These breeds included Australian Silky Terrier, Boston Terrier, Boxer, British Bulldog, Bullmastiff, Cavalier King Charles Spaniel, Chihuahua, Dogue de Bordeaux, French Bulldog, Havanese, Papillon, Pug, Rottweiler, Shar Pei, Shih Tzu, Staffordshire Bull Terrier and Tibetan Spaniel [22–26].

Table 1. Dog breeds represented in the survey.

Group 1: Toys	N	%	Group 2: Terriers	N	%	Group 3: Gundogs	N	%	Group 4: Hounds	N	%
Australian Silky Terrier *	2	0.7	American Staffordshire Terrier	1	0.4	Brittany	1	0.4	Afghan Hound	2	0.7
Cavalier King Charles Spaniel *	4	1.5	Bull Terrier	2	0.7	Cocker Spaniel	8	2.9	Basenji	5	1.8
Chihuahua *	1	0.4	Bull Terrier Miniature	1	0.4	Field Spaniel	2	0.7	Beagle	3	1.1
Chinese Crested Dog	1	0.4	Jack Russell Terrier	2	0.7	Flat Coated Retriever	1	0.4	Borzoi	1	0.4
Havanese *	2	0.7	Scottish Terrier	2	0.7	German Shorthaired Pointer	5	1.8	Dachshund (Min. Long)	1	0.4
Italian Greyhound	6	2.2	Soft Coated Wheaten Terrier	1	0.4	Golden Retriever	15	5.5	Dachshund (Min. Smooth)	1	0.4
Miniature Pinscher	2	0.7	St. Bernard	1	0.4	Gordon Setter	1	0.4	Deerhound	1	0.4
Papillon *	2	0.7	Staffordshire Bull Terrier *	17	6.2	Hungarian Vizsla	1	0.4	Petit Basset Griffon Vendeen	1	0.4
Pug *	5	1.8	Tenterfield Terrier	1	0.4	Hungarian Wirehaired Vizsla	1	0.4	Rhodesian Ridgeback	3	1.1
Tibetan Spaniel *	2	0.7	West Highland White Terrier	5	1.8	Labrador Retriever	12	4.4	Saluki	4	1.5
						Nova Scotia Duck Tolling Retriever	3	1.1	Whippet	6	2.2
						Weimaraner	2	0.7			
						Welsh Springer Spaniel	1	0.4			
Total Group 1	27	9.9	Total Group 2	33	12.0	Total Group 3	53	19.3	Total Group 4	28	10.2

Table 1. *Cont.*

Group 5: Working Dogs	N	%	Group 6: Utility	N	%	Group 7: Non-Sporting	N	%
Australian Cattle Dog	1	0.4	Alaskan Malamute	3	1.1	Boston Terrier *	1	0.4
Australian Kelpie	6	2.2	Boxer	6	2.2	British Bulldog	2	0.7
Australian Shepherd	6	2.2	Bullmastiff	2	0.7	Dalmatian	1	0.4
Belgian Shepherd Dog	7	2.6	Cane Corso	2	0.7	French Bulldog *	1	0.4
Border Collie	13		Dobermann	2	0.7	German Spitz	1	0.4
Collie (Rough)	2	0.7	Dogue de Bordeaux *	1	0.4	Great Dane	7	2.6
Collie (Smooth)	1	0.4	German Pinscher	1	0.4	Japanese Spitz	1	0.4
Finnish Lapphund	2	0.7	Leonberger	1	0.4	Poodle (Miniature)	3	1.1
German Shepherd Dog	13	4.7	Neapolitan Mastiff	1	0.4	Poodle (Standard)	7	2.6
Maremma Sheepdog	1	0.4	Newfoundland	2	0.7	Poodle (Toy)	2	0.7
Shetland Sheepdog	2	0.7	Old English Sheepdog	2	0.7	Schipperke	1	0.4
Welsh Corgi (Cardigan)	1	0.4	Portugese Water Dog	1	0.4	Shar Pei *	1	0.4
Welsh Corgi (Pembroke)	3	1.1	Pyrenean Mountain Dog	1	0.4	Shih Tzu *	1	0.4
			Rottweiler *	10	3.6	Tibetan Terrier	1	0.4
			Russian Black Terrier	2	0.7	Xoloitzcuintle	2	0.7
			Samoyed	1	0.4			
			Schnauzer	1	0.4			
			Siberian Husky	3	1.1			
			Tibetan Mastiff	1	0.4			
Total Group 5	58	21.2	Total Group 6	43	15.7	Total Group 7	32	11.7

* Brachycephalic dog breed (Brachycephalic dog breeds are those which have a facial skeleton relatively shorter than the cranial cavity [17]).

3.2. Principal Components Analysis Relating to the Dam

The 23 items relating to the dam were reduced using principal components analysis (PCA). Prior to performing PCA, the appropriateness of the data for analysis was assessed. The correlation matrix revealed the presence of many coefficients of 0.30 and above, the Kaiser–Meyer–Oklin exceeded the recommended value of 0.60 at 0.87, and Bartlett's Test of Sphericity reached statistical significance, supporting the factorability of the correlation matrix. Five components were presented with an eigenvalue exceeding 1, explaining 64.06% of the total variance. After observing the rotated component matrix, four questions were removed due to either a component loading lower than 0.55 ("good cut off loading" i.e., "my dam is confident" and "my dam has an outstanding pedigree") or too few items within the component (i.e., component 5 contained two items: "my dam has outstanding conformation according to the breed standard" and "my dam is within the accepted size according to the breed standard") [21]. This resulted in 19 items being retained (Table 2) and four components remaining which explained 63.23% of the total variance.

Table 2. Loading for the 19 items of the dam scales generated by means of Principal Component Analysis.

	Component			
Survey Question	**1**	**2**	**3**	**4**
My dam has naturally conceived with ease	0.733			
My dam has whelped with ease	0.803			
My dam has an excellent maternal instinct towards her puppies	0.729			
My dam produces sufficient milk to raise her puppies	0.762			
I would not breed from a dam if conception and whelping were difficult	0.675			
I would not breed from a dam if her maternal behaviour was not ideal	0.625			
I would not breed from a dam if her puppies did not conform closely to the breed standard		0.705		
I would not breed from a dam if the temperament of her puppies was not ideal		0.761		
I would not breed from a dam if she had rejected her puppies		0.567		
I would not breed from a dam if some of her puppies had a significant genetic fault		0.735		
I would not breed from a dam that was aggressive towards unknown people		0.605		
My dam is excitable			0.617	
My dam is obedient			0.806	
My dam has a strong bond to humans			0.659	
My dam is trainable			0.761	
My dam has an optimal temperament for the breed				0.747
My dam has passed all required and recommended health tests for her breed				0.735
My dam comes from a line of healthy, long lived relatives				0.658
My dam is friendly to dogs, other animals and people				0.604

The four components were labelled: Maternal Care (Component 1), Offspring Potential (Component 2), Dam Temperament (Component 3) and Dam Genetics and Health (Component 4). Component 1 was labelled Maternal Care as all questions within this component related to conception, whelping and the dam's ability to raise and care for her puppies. Offspring Potential was labelled for Component 2 as the questions related to the offspring's look and temperament. This Component also included the potential for temperament and genetic traits to be passed on to future generations. For example, if the dam is aggressive or rejects her puppies it may be likely that her offspring will do the same. Dam Temperament was considered for Component 3 as all questions within this component related to the dam's behaviour. Component 4 was labelled Dam Genetics and Health as questions within this component related to health and genetically driven behaviours. The lower the score, the more the respondents found the component to be important. There were six questions related to maternal care with a range of 6–30; five questions related to offspring potential with a range of 5–25; and both genetics/health and temperament contained four questions each with a range from 4 to 20 for each component. Cronbach's alpha for these components were 0.861, 0.747, 0.741, and 0.718 respectively.

3.3. General Characteristics of the Breeders

The majority of respondents were females (88%) and were aged between 18 and 85 years (n = 270: mean = 50.6, SD = 13.4). Most of the participants lived in New South Wales (41.6%) followed by South Australia (24.1%), Queensland (11.7%), Victoria (9.1%), Western Australia (8.8%) and Tasmania (2.6%). Six respondents (2.2%) did not report the state they were from. Half of the respondents had completed either high school (25.9%) or Technical and Further Education (TAFE) (25.9%). Some participants had completed an undergraduate degree (16.4%) with another 72 respondents (26.3%) completing a postgraduate degree. Of the remaining respondents, 5.1% had completed something other than that described, such as a diploma or a trade.

The most common dog breed within the survey was the Staffordshire bull terrier (n = 17, 6.2%). Participants were most likely to breed only one breed of dog (76.6%), with some participants breeding two (19.3%) or three breeds of dog (4.0%). Around one third (33.5%) had been breeding between 0 and 9 years, with the remaining having bred for more than 10 years (Table 3). Most breeders owned dams and dogs (80.3%), however 39 breeders (14.2%) only owned bitches, and 11 breeders (4.0%) only owned dogs. The remaining breeders (n = 4) owned neither dogs nor bitches. Almost all respondents (n = 267 or 97.4%) were part of the ANKC. Of the respondents who were not part of the ANKC (1.8%, n = 5), one breeder was associated with a working dog association recognised by the ANKC and another breeder was a member of a breed group/club.

Table 3. Number of years dog breeders had bred and/or owned their dogs.

	No. of Years Breeding Dog		No. of Years Owning Dog	
Years	**N**	**%**	**N**	**%**
<5 years	59	21.5	13	4.7
5–9 years	33	12.0	38	13.9
10–19 years	65	23.7	73	26.6
20–29 years	51	18.6	70	25.5
30–39 years	32	11.7	53	19.3
40+ years	33	12.0	27	9.9

3.4. Dam Breeding Priorities and ANKC Breed Group

The importance of maternal care differed significantly between ANKC breed groups (F (6, 264) = 2.41, p = 0.028; partial η^2 = 0.05). The Toy and Hound dog breeding groups scored Maternal Care significantly more important than the Terriers, Gundogs, Working dogs and Utility groups. The Non-sporting dog breeding group scored maternal care significantly more important than the Utility breeds. Other factors were not significant for breed group: Offspring Potential (F (6, 264) = 1.69, p = 0.123; partial η^2 = 0.04), Dam Temperament (F (6, 264) = 1.14, p = 0.341; partial η^2 = 0.03) and Dam Genetics and Health (F (6, 264) = 1.59, p = 0.150; partial η^2 = 0.04) (Table 4).

3.5. Breeding Priorities Relating to the Dam and the Number of Litters Produced

Two hundred and forty-two (89.3%) respondents bred two litters or less a year, while 29 breeders (10.7%) bred more than two litters per year. Components were not significantly different compared to the number of litters produced per year: Maternal Care (F (1, 267) = 3.09, p = 0.080; partial η^2 = 0.07), Offspring Potential (F (1, 267) = 0.02, p = 0.892; partial $\eta^2 \leq 0.01$), Dam Temperament (F (1, 267) = 0.05, p = 0.817; partial $\eta^2 \leq 0.01$) and Dam Genetics and Health (F (1, 267) = 2.02, p = 0.156; partial η^2 = 0.03).

3.6. Breeding Priorities in Relation to the Dam and the Number of Dog Breeds

Seventy-six percent of breeders bred one dog breed (n = 207). Dam Temperament was significantly affected by the quantity of breed types that the breeder bred (F (2, 268) = 3.17, p = 0.044; partial η^2 = 0.07) (Table 5). The breeders of one breed type placed more importance on Dam Temperament compared to breeders who bred two types of breed. There were no significant differences between breed number and the other factors: Maternal Care (F (2, 268) = 1.15, p = 0.317; partial η^2 = 0.03), Offspring Potential (F (2, 268) = 2.36, p = 0.097; partial η^2 = 0.04), and Dam Genetics and Health (F (2, 268) = 0.91, p = 0.403; partial η^2 = 0.01).

Table 4. Principal Component Analysis subscale relating to the Dam according to ANKC breed group. Lower values represent higher importance.

						95% Confidence Interval	
Outcome Variable	**df**	**Df Error**	***F***	**Breed Group**	**Means**	**Lower Bound**	**Upper Bound**
Maternal care	6	911.73	2.408	Toys	0.916	0.858	0.974
				Terriers	0.986	0.934	1.038
				Gundogs	0.977	0.937	1.018
				Hounds	0.906	0.850	0.962
				Working dogs	1.000	0.962	1.038
				Utility	1.011	0.965	1.057
				Non-Sporting	0.959	0.907	1.011
Offspring Potential	6	911.73	1.693	Toys	0.818	0.767	0.868
				Terriers	0.860	0.814	0.905
				Gundogs	0.855	0.820	0.891
				Hounds	0.827	0.778	0.875
				Working dogs	0.893	0.859	0.926
				Utility	0.845	0.805	0.885
				Non-Sporting	0.825	0.780	0.871
Dam Temperament	6	911.73	1.138	Toys	0.913	0.857	0.969
				Terriers	0.929	0.878	0.979
				Gundogs	0.923	0.884	0.962
				Hounds	0.993	0.939	1.047
				Working dogs	0.937	0.899	0.974
				Utility	0.964	0.919	1.008
				Non-Sporting	0.934	0.883	0.984
Dam Genetics and Health	6	911.73	1.591	Toys	0.674	0.631	0.718
				Terriers	0.702	0.663	0.742
				Gundogs	0.678	0.647	0.708
				Hounds	0.747	0.705	0.789
				Working dogs	0.704	0.675	0.733
				Utility	0.691	0.657	0.726
				Non-Sporting	0.675	0.636	0.715

Table 5. Breeder priorities relating to the Dam and the number of breeds that the participant bred.

						95% Confidence Interval	
Outcome Variable	**df**	**Df Error**	***F***	**Number of Breeds**	**Means**	**Lower Bound**	**Upper Bound**
Maternal care	2	530	1.154	1	0.965	0.945	0.986
				2	1.000	0.959	1.042
				3	0.988	0.894	1.083
Offspring	2	530	2.357	1	0.847	0.829	0.864
				2	0.859	0.823	0.894
				3	0.937	0.856	1.018
Dam Temperament	2	530	3.168	1	0.929	0.909	0.949
				2	0.985	0.946	1.024
				3	0.946	0.857	1.036
Dam Genetics and health	2	530	0.911	1	0.691	0.675	0.706
				2	0.704	0.673	0.735
				3	0.734	0.664	0.805

3.7. Breeding Priorities Relating to the Dam and Brachycephalic Dog Breeds

There were 54 breeders (19.9%) with brachycephalic dogs. Offspring Potential (F (1, 269) = 5.14, p = 0.024; partial η^2 = 0.09) and Dam Genetics and Health (F (1, 269) = 4.33, p = 0.038; partial η^2 = 0.06) significantly differed when comparing whether the dog breed was brachycephalic or not. Breeders of brachycephalic dogs scored Offspring Potential and Dam Genetics and Health significantly more important than breeders of non-brachycephalic dogs. There were no significant differences for non-brachycephalic and brachycephalic breeds in Maternal Care (F (1, 269) = 0.09, p = 0.771; partial η^2 < 0.01), and Dam Temperament (F (1, 269) = 1.51, p = 0.221; partial η^2 = 0.03) (Table 6). Seven breeders bred at least two brachycephalic dog breeds (2.6%) while 26 of the 63 breeders breeding more than one breed type bred at least one brachycephalic dog breed (9.5%).

Table 6. Breed priority according to whether the dog breed was brachycephalic.

						95% Confidence Interval	
Outcome Variable	**df**	**Df Error**	***F***	**Brachycephalic Breed**	**Means**	**Lower Bound**	**Upper Bound**
Maternal care	1	266	0.085	No	0.974	0.954	0.995
				Yes	0.968	0.927	1.008
Offspring	1	266	5.138	No	0.861	0.844	0.879
				Yes	0.816	0.781	0.851
Dam Temperament	1	266	1.505	No	0.946	0.926	0.965
				Yes	0.919	0.880	0.958
Dam Genetics and health	1	266	4.326	No	0.702	0.687	0.717
				Yes	0.666	0.636	0.697

3.8. Sire Selection

Twenty-nine percent of breeders accessed a distant sire owned by someone else, 23.7% of breeders used their own sire, while accessing a local sire for breeding was less common (9.9%). For breeding, the breeders' own sire or a local sire was most commonly used (35.4%), however others imported frozen semen (6.2%) or imported the sire (0.7%). The number of breeders opting to use artificial insemination was rather small in this dataset (7.3%). Many breeders (67.8%) spent time interacting with the sire before selection but 28.0% of breeders indicated that interaction was not possible. Variables affecting sire selection are displayed in Table 7. Breeders rated the sire's conformation and temperament highly, together with his ability to produce healthy puppies and complementing the dam.

Table 7. Variables important to the selection of the sire.

	Respondents (%)						
Variable Importance	**Location**	**Conformation**	**Size**	**Pedigree**	**Temperament**	**Complements Dam**	**Healthy Puppies**
High	6.0	95.5	84.7	74.3	98.9	99.6	95.5
Neutral	10.9	3.7	14.6	20.5	1.1	0.0	3.7
Low	83.0	0.7	0.7	5.2	0.0	0.4	0.7

3.9. Physical and Genetic Testing of Both Dams and Sires

If more than five respondents represented a single breed, they were identified for physical and genetic tests conducted. There were 11 dog breeds where this occurred (Table 8). Some breeders undertook more tests than others and many of the breeders undertook tests specific to their breed. For example, DNA testing for Neuronal Ceroid Lipofuscinosis (NCL) was undertaken by all 12 Border collie breeders while 11 out of the 14 Golden Retriever breeders undertook hip scoring.

Table 8. Genetic and Physical testing of common dog breeds within the survey.

Breed	Breeders (*n*)	DNA Test	Breeders Undertaking Test (*n*)	Physical Tests	Breeders Undertaking Test (*n*)
Basenji	5	Fanconi	5	Eye assessment	5
		Progressive Retinal Atrophy	4	Hip score	2
		Hemolytic anaemia	2	Thyroid	2
		Pyruvate kinase deficiency	1	Heart assessment	1
		DNA inbreeding coefficient Factor 7	1		
		DNA identification Thyroid	1		
Belgian Shepherd Dog	5	Colour MDR1 masking	1	Hip score	3
				Elbow score	3
				Eye assessment	2
				Heart assessment	7
Border Collie	12	Neuronal Ceroid Lipofuscinosis	12	Elbow score	11
		Trapped Neutrophil Syndrome	11	Hip score	10
		Collie Eye Anomaly	10	Eye assessment	6
		Multi-Drug Resistance Gene 1	2	General vet check	1
		Imerslund-Grasbeck Syndrome	2	Chiropractor vet check	1
		Degenerative Myelopathy	1	Collie collapse	1
		Parentage (Orivet)	1	Hearing test	1
		Glaucoma	1		
		B12	1		
German Shepherd Dog	11	Degenerative Myelopathy	6	Hip score	6
		Ivermectin Sensitivity	2	Elbow score	6
		Long stock coat gene	1	X-ray (not specified)	1
		Canine renal Dysplasia	1	Vet check	1
		Dwarfism	1		
		Haemophilia	1		
Golden Retriever	14	Ichthyosis	12	Hip score	11
		Progressive Retinal Atrophy 1	10	Eye assessment	11
		Progressive Retinal Atrophy 2	10	Heart assessment	11
		Progressive Rod Cone Degeneration	4	Elbow score	10
				Dentition assessment	1

Table 8. *Cont.*

Breed	Breeders (*n*)	DNA Test	Breeders Undertaking Test (*n*)	Physical Tests	Breeders Undertaking Test (*n*)
Great Dane	5	Heart testing	5	Hip score	5
		Thyroid	4	Elbow score	5
		Colour DNA	1	Shoulder and neck X-rays	1
Labrador Retriever	11	Progressive Retinal Atrophy	10	Hip score	5
		Exercise-induced Collapse	8	Elbow score	5
		Progressive Rod Cone Degeneration	2		
		Coat colour	1		
		Long hair	1		
		DNA identification	1		
		Centronuclear Myopathy	1		
Poodle (Standard)	5	Degenerative Myelopathy	3	Eye assessment	4
		Neonatal Encephalopathy	3	Hip score	4
		von Willebrand's disease	3	Skin biopsy	1
		Thyroid	2	Vet checked	1
		Full DNA data	1		
		Progressive Retinal Atrophy	1		
		Renal Dysplasia	1		
Rottweiler	8	DNA testing	1	Hip score	8
		von Willebrand's disease	1	Eye assessment	8
				Dentition assessment	8
				Elbow score	7
				Heart assessment	3
				Joint assessment	1
				Brachycephalic Obstructive Airway Syndrome	1
Staffordshire Bull Terrier	16	Hereditary Cataracts	12	Eye assessment	7
		L2-Hydroxyglutaric aciduria	12	Hip score	3
		Full DNA test	2	Heart assessment	2
		Coat colour	1	Elbow score	1
				Dentition assessment	1
				X-ray (not specified)	1
				Vet check	1
West Highland White Terrier	5	Genetic technologies	1		

4. Discussion

This study aimed to gain insight into breeding stock selection of Australian purebred dog breeders, with a particular emphasis on dams. We discuss the general characteristics and breeding priorities of a small sample of Australian purebred dog breeders covering 91 different breeds across seven breed groups classified by the ANKC. The majority of 274 breeders surveyed bred only one dog breed, kept three and two bitches and dogs respectively, and bred two litters or less a year. The implications of this study include the potential to provide the findings to dog breeding groups and governing bodies which may endorse important breeding priorities and thus produce improved dog litters.

4.1. The Impact of the Number of Litters Produced and the Number of Dog Breeds on Breed Priority Relating to Dams

In our sample of active and non-active breeders, almost three quarters (69%) produced one litter or less per year, which is slightly above those presented by the ANKC (54%) [3]. However, we found fewer breeders breeding 5–10 litters per year (2% compared to 5% respectively). Consistent with Leroy et al. [4] in a population of French breeders, we found that the number of litters produced was not significantly associated with breed priority. It appears that the larger kennels (determined by the number of breeding bitches and litters produced) within the current study are observant of their breeding dogs, and prioritise the health and wellbeing of their animals to a similar extent to smaller kennels. We did however, find that the breed related to the priorities and practices of the breeder. For instance, breeders of hunting and working dogs produce less stock as they are breeding to satisfy their own needs and replenish their working stock [4]. While breeders of working stock may be breeding for their own purpose, breeders of increasingly popular dog breeds, such as the Pug [27], may be producing stock for companionship and therefore litters produced would be higher.

It was expected that Dam Temperament would feature as a component from the principal component analysis given that there is a large body of work investigating temperament in dogs (e.g., [28–30]). Unlike the number of litters produced per year, breeders that bred a single breed rated Dam Temperament as significantly more important than breeders that bred more than one breed. Statements in this component included the dam being excitable, obedient, having a strong bond to humans, and trainable. A possible reason for the differences observed between the breeds may be due to the different dog breeds which vary in their levels of excitement, obedience and trainability. Thus, the breeder may not necessarily share the same focus across multiple breeds, which would then reduce the priority of Dam Temperament. Research into the impact of the number of breeds kept and the impact on Dam (as well as sire) Temperament is currently lacking, and needs to be investigated further.

4.2. Breeder Priorities in Relation to Maternal Care

Of the four breed priorities relating specifically to dams identified through PCA, questions relating to breeding and dam-puppy interactions were identified. Studies of maternal care behaviour in species such as rats, dogs and humans have shown that maternal care can have implications for the behaviour of young later in life, particularly in relation to their response to stressful events [12–14]. Maternal care is critical for the survival of altricial animals, where young are born immature (deaf, blind) and rely solely on their mother for survival [14,31]. Priorities relating to Maternal Care significantly differed between ANKC dog breed group, suggesting that it might be more relevant for some, but not all breeds. For instance, Maternal Care was a higher priority for breeders of Toy and Hound ANKC groups compared to the Terrier, Gundog, Working dog and Utility groups. The Maternal Care component was a mixture of statements, including conceiving and whelping with ease, as well as maternal instinct and milk production.

The majority of brachycephalic dogs consisted of breeds from the Toys and Non-sporting ANKC groups. It is common for brachycephalic dams to experience dystocia [15–17], difficulty of birthing the puppies naturally through the birth canal [27,32], and forcing the dam to have a caesarean birth. Bitches requiring caesareans due to dystocia account for more than 60% of births [15,33,34].

Maternal care may be impaired in dams recovering from surgery due to a caesarean birth, causing the breeder to be more involved with the litter. The dam is needed within the litter for puppy survival and development. The puppies not only feed from their mother when very young, but are also influenced by her temperament and behaviour. Further understanding of breed priorities according to the brachycephalic index would allow targeted implementation of breed standards and criteria. While comparative data on conception and prevalence of caesarean sections is lacking across all dog breeds, a recent study highlighted a general reduction in the fertility of male dogs [35]. In a retrospective cohort study of Norwegian purebred dogs, 8% of pups died before eight days of age, in 10,810 litters and 224 breeds [36]. As well as the age of the bitch and litter size, breed was also an important factor influencing perinatal mortality, although the largest variation was between litters. In breeds that experience problems in conception, birthing type of perinatal mortality, it is likely that the Maternal Care component would score more highly. For example, lack of maternal instincts may mean breeders have to work much harder to keep the puppies alive, and if early colostrum is not received by the puppies, then they will be less likely to thrive [37]. We recommend that future studies focus on questions relating specifically to maternal care and birthing method to determine the true influences on breeders' choices in this area. An example of an important initiative in this area is the veterinary reporting of caesareans and procedures to alter the natural conformation of dogs being supported in the UK by the Kennel Club, British Veterinary Association, British Small Animal Veterinary Association and Royal College of Veterinary Surgeons [38]. Of course, we acknowledge that it is not possible to select for dam maternal behaviour until it has been observed at least once. So, although it will not influence the initial decision to breed a bitch, it should be considered for subsequent breedings.

4.3. Breeder Priorities in Relation to Genetics and Health

There are a large number of hereditary diseases identified in the dog population, second to humans [39]. This allows dog diseases to be identified and possibly treated in several breeds. Over 350 inherited disorders have been categorised by the American Kennel Club [7]. Of these diseases, many are restricted to specific breed groups or particular breeds [8], such as syringomyelia in the Cavalier King Charles spaniel [8]. Some diseases affect a large majority of dog breeds, e.g., hip and elbow dysplasia [40–42], and their heritability and incidence are continually being revised and reported. Reflective of this, breeders surveyed in this study rated Dam Genetics and Health as the most important component when selecting dams, and actively conducted genetic and physical testing of their dogs. Brachycephalic dog breeders gave even higher importance to Dam Genetics and Health than breeders of non-brachycephalic dog breeds. It is assumed that breeders of brachycephalic dogs are aware of the problems associated with these breeds of dogs (e.g., obstructive airway syndrome (BOAS) is a common constraint for brachycephalic breeds [43]), and therefore place more importance on additional genetic and health aspects which may also be present. Genetic testing was more relevant to some breeds than others, likely reflecting known issues within the breed. For example, all of the Rottweiler breeders assessed their dogs for dentition assessment, indicating that this is an important problem for the breed. Eight breeds were assessed for elbow scoring. Indeed, elbow dysplasia and borderline signs were observed in half of the Rottweilers included in an official screening program (see [44]).

Important diseases have been recorded in dogs and by all breeders (http://discoveryspace.upei.ca/cidd/breeds/overview). A review of common disorders inherited in purebred dogs is also available [18]. In purebred dogs, in a case-control study for 24 common hereditary disorders, 10 disorders were found to be more prevalent than in mixed-breed dogs, suggesting a greater proportion of diseases occurring in purebred than in mixed-breed dogs [45]. Multiple disorders are associated with brachycephalic breeds: BOAS in Boston Terriers and Pugs [46], dystocia in Bull Terriers [27], eye problems in Pugs and Shar Peis, and mitral valve disease in Cavalier King Charles Spaniels [27]. Only one breeder of six described undertaking a breathing test for their pug, although 67% (n = 6) documented eye testing. While no common tests were undertaken by Boston Terrier (n = 2) and Shar Pei breeders (n = 1), almost all breeders of Cavalier Kind Charles Spaniels (6/7) had hear auscultations as a physical test undertaken on their dogs. It seems important to highlight that a common disease test for the Pug is not being undertaken, and needs further investigation as to the reasons behind this. Although Dam Genetics and Health were deemed more important by brachycephalic breeders, it seems that they are not always undertaking the relevant tests. In the current dataset, all Great Dane breeders tested for dilated cardiomyopathy and almost all of Rottweiler breeders tested for elbow dysplasia. The top ten significant disorders of the ANKC include hip dysplasia, epilepsy, hypothyroidism, allergies, hemangiosarcoma, patella luxation, cataracts, lymphoma, bloat and progressive retinal atrophy [47]. Of these important diseases listed by the ANKC, all of the Great Dane breeders tested for hip dysplasia. However, even though hip dysplasia is a common problem in Labrador Retrievers and German Shepherds [48], only around half of breeders reported using Hip Scores on their breeding dogs. This may have been due to not all breeders completing this section of the survey, as registered breeders of Labrador Retrievers must have their breeding dogs hip scored, although this is not mandatory for German Shepherd breeders in Australia [49,50]. Further investigation into the reasons behind decisions to undertake genetic and health testing are required. Such knowledge is likely to help the efforts to increase the number of breeders undertaking tests on their breed stock.

4.4. Sire Selection

Only a small portion of the breeders surveyed used their own sires. When seeking sires, breeders preferred "distant" over "local" sires, and where possible, still preferred to meet and interact with the sire. Travelling distances to find sires might be required to ensure genetic diversity, which is particularly important for closed breeding lines. The location of the sire was a low priority for breeders, but conformation, size, pedigree, temperament, that the sire complements the dam, and the sire produces healthy puppies were all considered important in their decision. Only a small percentage chose to use artificial insemination methods. There are both advantages and disadvantages of artificial insemination (fresh or frozen semen) and natural mating in dogs. Advantages include an increased level of hygiene [9] and safety, as well as long term storage and the high number of usage per sire [51]. Natural mating in dogs may result in reduced whelping rates compared to artificial insemination using frozen semen [10]. Disadvantages of artificial insemination exist and include a limited shelf life, expensive to store and disruption of sperm numbers [51]. Artificial insemination using frozen semen results in a smaller litter size compared to artificial selection using fresh semen [52,53]. Artificial insemination may lessen the likelihood that the dam would become sick or injured during impregnation, allowing a more successful pregnancy due to reduced stress. If not already, details surrounding artificial means should be discussed between breeders and veterinarians given the animal welfare implications for the bitch.

4.5. Limitations and Future Work

There were a number of limitations of the survey, particularly the low number of breeders who participated. Given the nature of the survey where participants self-selected, non-response bias cannot be calculated however, the results may be representing participants who were responsible and conscientious breeders registered with the ANKC. Thus, the findings reported here do not necessarily reflect the priorities or practices or all purebred breeders across Australia, nor represent all of the different breeds of dogs. It is equally important to understand and compare the practices of non-registered Australian dog breeders [54] as they are likely to differ in their breeding practices.

A number of important questions were unintentionally omitted from this study. For example, future studies should ask for the number of puppies born alive per year, whether it was more likely for the dam/(s) to give birth naturally or by caesarean, and other factors which may be important for specific breeds (i.e., hunting/retrieving capabilities for gundogs). Some issues were discovered with several questions that could also be improved for future studies. For example, our questions relating to priorities and practices were not breed specific, which did not allow breeders that bred more than one breed to be distinguished. In relation to accessing sires, there was some confusion as to the definition of *distant sire* given what people constitute as "distant", and their willingness to travel (or import semen) are likely to vary among breeders and especially breeders of rare breeds. The survey also highlighted that some breeders wanted to undertake physical or DNA testing for their dogs but there were reasons as to why this was not conducted. For some, it was not physically possible (i.e., the breeder was in a remote location), tests were unavailable within Australia or there were no specific tests available. The expense of such testing may also influence decisions. We recommend that those involved with dog breeding and breed maintenance (i.e., veterinarians, ANKC) discuss options that are available to breeders so such tests can be accessed.

5. Conclusions

This study represents a step toward understanding breeding stock selection and breed priorities of Australian purebred dog breeders. Emphasis was given to breeding priorities and practices surrounding the Dam, given the important role maternal care has on the development of puppies. These findings provide useful insight into dog breeding, and provide information that may be helpful for dog breeding groups and governing bodies (such as state governments, the ANKC) to manage breeding and breeder education. For example, the impact of caesarean births on mothering ability could be addressed to highlight the importance the mother has on puppy health and behaviour throughout the puppy's life. A significant association between ANKC breed group, the number of dogs the kennel bred, and whether the breed was brachycephalic on breed priority was found. This suggests that a "one size fits all" model for selecting and managing breeding stock is not appropriate. Importantly, it seemed that many breeders did not prioritise the maternal care behaviour of the dams. Emphasis of maternal care as a selection factor (for subsequent breeding of a bitch) should be made more prominent to breeders due to the impact it may have on the puppy's stress-related behaviour later in life.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2615/6/11/75/s1, Breeder Survey questions.

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